Source and Carrier Effect on the Bioactivity of BMP Bio-Implants

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science

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Abstract

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Master of Science 2013
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Bone morphogenetic protein-2 (BMP-2) plays a critical role in bone formation.

The aim of this study was to compare the activity of the mammalian cell BMP-2 to the E-coli cell BMP-2.

In vitro, the potency of mammalian and E-coli BMP-2 was compared by adding BMP-2 to C2C12 cells and measuring the level of alkaline phosphatase activity.
In vivo, the activity was evaluated by placing the bioimplants in the thigh muscle of mice, and measuring the amount of bone induced.

The in vitro assay clearly showed that mammalian BMP was significantly more potent than E-coli BMP. In vivo, on the calcium phosphate carrier, mammalian BMP produced more bone than E-coli BMP, but E-coli BMP produced higher density tissue than mammalian BMP. On both mammalian and E-coli BMP, the calcium phosphate carrier had a significant effect on the density but not the quantity of bone produced versus the absorbable collagen sponge carrier.

Dedication

“It is hard to fail, but it is worse never to have tried to succeed.”

— Theodore Roosevelt

This work is dedicated to all of those who have tried to succeed in the past, and to those who will do the same in the future. My successes are yours to share.

Acknowledgments

I wish to acknowledge the mentors who have guided me through the program and my masters' project.

Dr.Cameron Clokie was of pivotal importance and support during the writing and development of this thesis, and Dr. Grace Bradley provided timely and constructive feedback along the way.

Acknowledgement goes out to Aileen Zhou for offering her help in the statistical analysis of the data. In addition, I would like to underline the hard work and support of Zhining Zhu for his help in the laboratory experiments and the poster presentation.

Nancy Valiquette was of great aid as a surgical assistant, with the histological analysis of the specimens, and with the interpretation of the microCT slides.

I would like to extend a special thank you to Dr. Sean Peel for providing guidance from the start, assisting in designing the experiments, and for his timely constructive feedback.

I would like to extend my humble acknowledgements to my fellow colleagues, who have enabled for this research to take place, by providing time and support when it was needed.

Finally, an acknowledgement is made to my partner, and best friend, Dr. Marc DuVal, who has stirred me in the right direction during the growth of this project.

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List of Abbreviations

ACS: absorbable collagen sponge

ADU: arbitrary density unit

ALK: activin receptor-like kinase

ALP: alkaline phosphatase

ANOVA: analysis of variance

Arg: arginine

Asn: asparginine

BCP: biphasic calcium phosphate

BMA: bone marrow aspirate

BMC: bone mineral content

BMD; bone mineral density

BMP: bone morphogenetic protein

BMPR: bone morphogenetic protein receptor

BSC: biologic safety cabinet

BV: bone volume

BVF: bone volume fraction

CaP: calcium phosphate

CBF: core binding factor

cDNA: complementary DNA

CHO: Chinese hamster ovary cells

CPC: calcium phosphate-based cements

CT: computed tomography

DBM: demineralised bone matrix

DEXA: dual-energy xray-absorptiometry

DNA: deoxyribonucleic acid

Dpp: decapentaplegic protein

E: E-coli BMP (Cowell Medi)

ECM: extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: epithelial growth factor

ELISA: Enzyme-linked immuno sorbent assay

ER: endoplasmic reticulum

FBS: fetal bovine serum

FGF: fibroblast growth factor

GH: growth hormone

HA: hydroxyapatite

HU: Hounsfield units

IGF: Insulin-like growth factor

IL: interleukin

KDa: kilodalton

M1: mammalian BMP (Induce)

M2: mammalian BMP (Medtronic Infuse)

αMEM: α-minimal essential medium

mRNA: messenger ribonucleic acid

MSC: mesenchymal stem cell

NaOH: sodium hydroxide

OP1: osteogenic protein 1

OSE: osteoblast specific element

PBS: phosphate buffered saline

PDGF: platelet derived growth factor

pNP: p-nitrophenol

pNPP: p-nitrophenol phosphate

PTH: parathyroid hormone

rhBMP: recombinant human bone morphogenetic protein

ROI: region of interest

RUNX2: Runt-related transcription factor 2

Ser: Serine

Smad: small mothers against decapentaplegic

$T_{1/2}$: biological half life

TCP: tricalcium phosphate

TGF-ß: transforming growth factor beta

Thr: threonine

TMC: tissue mineral content

TMD: tissue mineral density

TNF: tumour necrosis factor

TV: total volume

VEGF: vascular endothelial growth factor

1 Introduction

Bone grafting techniques have been used by medical specialists for more than 100 years. Currently, more than 500,000 bone grafting procedures are performed yearly in the United States in the fields of dentistry, neurosurgery, and orthopaedics. Bone is the second most common transplanted tissue [1, 2].

When the opportunity for bony reconstruction is presented to the surgeon, many choices must be weighed before a proper graft material is chosen. Factors which must be considered include the site of reconstruction, size of the defect to repair, objectives of the surgery, desires of the patient, and knowledge of graft materials. There are many options in graft materials from which to choose.

Autogenous grafting is considered the gold standard for osseous skeletal repair and reconstruction of the maxillofacial complex. Autogenous bone provides osteoinductive growth factors, osteogenic cells, and an osteoconductive scaffold. Autogenous bone carries no risk of disease transmission or graft rejection. However, its limited availability, donor site morbidity, and unpredictability in graft resorption force the surgeon to consider other grafting materials for use in skeletal defects of the maxillofacial system.

Historically, alternatives to autogenous grafts have been allogeneic, xenogeneic and alloplastic materials. Ideal substitutes should be osteoconductive, osteoinductive, biocompatible, resorbable, structurally similar to bone, easy to use, and cost effective.

Allogeneic grafting involves use of human bone harvested from a genetically non-identical member of the same species as the host. Its use has increased significantly in the past decade and accounts for about one-third of bone grafts performed in the United States [2, 3]. Although allografts undergo various treatments after harvest to limit the potential for disease transmission, the risk is not entirely eliminated. Unfortunately, these treatments weaken the biologic and mechanical properties present in the initial bone tissue. Therefore, allogeneic grafts are not inherently osteogenic and function

primarily as a physical scaffold. Its osteoinductive capacity is highly variable depending on the processing method and sourcing[4].

Xenogeneic grafts are derived from a genetically different species than the host. Examples of these types of biomaterials for human use are coralline bone and bio-oss. They have the advantages of lower costs and no significant increase in operating time, nor patient morbidity. However, they pose a risk for foreign body reaction, graft rejection, and disease transmission [5]. Furthermore, xenografts may be subjected to a variety of treatments, including freeze-drying and deproteinization, in order to minimize their antigenicity and immune response. These treatments can lead to significant loss of biological and mechanical properties. Xenografts therefore function as osteoconductive matrices for osteoid production.

Alloplasts are manufactured synthetic bone material such as calcium sulphate and bioactive glasses. Alloplastic grafts function as an osteoconductive bioinert aid, providing a mechanical framework across which vascular ingrowth and osteoid production can occur.

Composite grafts combine an osteoconductive matrix with bioactive agents, therefore, adding the biological signal necessary for osteoinduction and osteogenesis.

Recently, there has been an interest in the application of growth factors as an adjunct to autogenous bone for the reconstruction of maxillofacial defects. Bone morphogenetic protein (BMP) has been the focus of much research and clinical development for this purpose.

1.1 Bone Graft Healing

Bone is a vital structure that constitutes part of the skeleton of vertebrates. It supports and protects the various organs of the body, produces red and white blood cells and acts as a storage reservoir for important minerals such as calcium and phosphate. Bone healing is a proliferative physiologic process by which the regeneration of tissue is performed through a complex interplay of cellular and

molecular events. Compared to other tissues, bone is unique in that it can heal entirely via regeneration [6]. Factors contributing to delayed or incomplete bone healing are systemic infection, insufficient immobilization, inadequate blood supply, use of corticosteroids and chronic disease[7]. In these circumstances, a bone graft is needed in order to aid in the repair of bone.

1.1.1 Autograft healing

Autogenous bone graft healing has been described as the process of envelopment and interdigitation of necrotic old bone with viable new bone [5, 8-10]. It is affected by several factors including contact with donor tissue, state of health of the recipient bed and the biomechanical properties of the graft [9]. In 1893, Barth originally coined the term "*schleichender Ersatz*" (German for *creeping substitution*) to the process by which fresh autogenous bone was replaced by host-invading bone without resorption [11]. Contrary to its original meaning, the term creeping substitution has been attached to a process by which incorporation of all grafts proceeds by the gradual resorption of the transplanted graft and replacement by new bone [8]. Bone graft healing is described in five phases with multiple phases overlapping or occurring simultaneously. The five phases include: inflammation, vascularization, osteoinduction, osteoconduction and remodelling [8-10, 12].

The first phase of autogenous bone graft healing is the inflammatory phase. This phase begins immediately during surgery and continues for the first one to two weeks post-operatively. During this initial phase, hematoma formation accompanies inflammation, and acts as a source for signalling molecules that have the capacity to initiate the cascade of cellular events critical to bone graft healing [13, 14]. Signalling molecules that play an important role in bone graft healing are pro-inflammatory cytokines (IL-1, IL-6, TNF-α), and growth and angiogenic factors (BMP, PDGF, FGF,TGF, IGF, and VEGF). Inflammatory cells, along with macrophages secrete inflammatory cytokines. These inflammatory cytokines recruit other inflammatory cells, stimulate angiogenesis, enhance extracellular matrix synthesis, and recruit fibrogenic cells to the site. In addition to the inflammatory cytokines, the damaged endothelium releases platelets that

undergo degranulation, releasing platelet-derived growth factor (PDGF), transforming growth factor-beta 1 and 2 (TGF-ß), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF). All of these growth factors have a powerful mitogenic and chemotactic effect on the surrounding tissues of the recipient bed [9, 15].

Surgical intervention and site preparation result in a recipient bed with compromised vascularity and a central hypoxic core [16, 17]. Placement of an inherently hypoxic graft into a recipient bed with compromised vascular support results in a local tissue environment that is hypoxic (oxygen tension approximately 3-10mm Hg), acidic (pH 4.0-6.0) and rich in lactate [18]. The contrasting environment set up between the normal adjacent tissue and the local hypoxic tissue bed stimulates circulating monocytes to differentiate into macrophages. Macrophages are activated via a number of avenues including bacterial and tissue factors, inflammatory cytokines (e.g. TNF-α) and the local environmental factors such as hypoxia, low pH and high lactate levels [17].

The combination of oxygen gradient, platelet degranulation, and release of the macrophage-derived factors culminates in stimulation of early angiogenesis from surrounding capillaries and mitogenesis of the transplanted osteocompetent cells [18]. The process through which an inflammatory response triggers vascular proliferation is essential to graft nutrition and cell survival.

The second phase of autogenous bone healing is revascularization and angiogenesis. The ingrowth of recipient blood vessels into the bone graft marks the beginning of graft resorption, and the beginning of new bone formation. In 1763, Haller first described the importance of vascularization in bone healing by stating: “ *the origin of bone is the artery carrying the blood and in it the mineral elements*” [19]. Revascularization of autogenous cancellous grafts can occur within hours of grafting, resulting in end-to-end anastomoses of the recipient vessels with those of the grafts. However, most investigators agree that clinically significant results are not seen before the third day after grafting [5, 10]. As early as day 3 following transplantation, capillary penetration into the graft allows osteocompetent cell proliferation. The capillaries perfuse the graft and decrease the oxygen gradient, leading to a mechanism that regulates angiogenesis. By day 7, blood borne macrophages start to secrete growth factors, and

this continues until revascularization of the graft is complete, which occurs between day 14 and 21. Local or systemic factors leading to inadequate vascularization can result in decreased bone formation and bone mass [20, 21]. Delayed revascularization of these grafts may be seen in situations such as infection or irradiation, resulting in reduced biological activity of the graft [22].

Vascularization provides oxygen and nutrients to the remaining viable osteocompetent cells, and MSC-like cells, retaining the ability of these cells to differentiate into multiple cell types, including osteoblasts [23, 24]. As these osteogenic cells induce nearby stem cells to differentiate into osteoblasts, osteoid matrix is laid down. These osteogenic cells come from either the host or the donor tissue [9]. Additionally, osteoclasts from the systemic circulation are delivered to the graft site. These osteoclasts resorb the original mineral matrix, thereby liberating entrenched BMP and IGF-1 and -2, which initiates the graft maturation[25] Further mesenchymal stem cell migration, proliferation and differentiation into osteoblasts may occur secondary to the release of BMP.[26]

Bone donor sites, such as the ilium and tibial plateau, are chosen for their ease of access and due to the cellular density of the cancellous bone. One of the key features of cancellous grafts is the osteocompetent nature of the endosteal osteoblasts, and the cancellous marrow stem cells. These two cell populations are transferred in viable states, surviving the first three to five days post transplantation largely due to their surface position and their ability to absorb nutrients from the recipient bed, until the graft becomes revascularized by capillary ingrowth. These cells can undergo an “out-of-body-experience” for up to four hours without losing more than 5% of their viability[18]. In bone marrow, mesenchymal cells are found at an approximate concentration of 1 in 50,000 to 1 in 1 million [27, 28]. Primitive mesenchymal cells present in bone marrow, and progenitors of endothelial cells are amongst the cells that are most resistant to ischemia after transplantation, and thus may survive in areas deeper in the graft tissue [29]. However, mature osteocytes do not survive the transfer, their mineral matrix being resorbed during the transplantation process. Although their nutritional supply is limited, surviving transplanted stem cells and endosteal osteoblasts are stimulated to produce a small amount of osteoid.

This leads to the third phase of autogenous bone healing called osteoinduction. Osteoinduction is a process whereby mesenchymal cells from the host are recruited to differentiate into osteocompetent cells. This recruitment and differentiation is largely mediated by polypeptides, such as BMP [8].

Osteoid is first laid down by remnant osteocompetent cells, such as endosteal osteoblasts, as a rim surrounding a core of necrotic bone [30]. The formation of new bone on a scaffold of graft trabeculae is responsible for the initial increase in radiodensity of bone grafted areas. This process begins at 2 weeks and continues for another 6 weeks. As osteoid is resorbed by osteoclasts, osteoblasts lay down new bone. The process of gradual laying down of immature bone and early remodeling may last for several months in cancellous grafts and longer in cortical grafts [31]. At this initial stage, the newly formed bone is hypocellular, more mineralized, and randomly organized [18] . The process of resorption of osteoid and formation of new bone is referred to as creeping substitution. It uses the fibrin network of the graft as a scaffold. This is referred to as osteoconduction [18]

Osteoconduction is considered the fourth phase of bone healing. It is defined as an ordered, spatial three-dimensional ingrowth of capillaries, perivascular tissue and osteoprogenitor cells from the recipient bed [8]. It is closely associated with vascularization and osteoinduction in the healing of autogenous bone grafts.

Remodelling is the final stage of bone graft healing, and begins with the arrival of osteoclasts. The process involves the resorption of the immature bone formed during the first phase as well as the trabeculae. These are replaced with mature, structurally organized lamellar bone. This lamellar bone is capable of withstanding normal occlusal functional forces. It is mature, load-bearing bone, and well-organized in its mineralized structure[18]. Remodeling occurs in conjunction with revascularization, osteogenesis, osteoinduction and osteoconduction. Approximately 4 months after graft placement, endosteum and periosteum develop [29, 30]. Integration into the recipient site is considered functional by 6 months, and is complete by one year post surgery [8]. Long term bony remodeling occurs throughout life in response to functional loads placed on the area (figure 1)

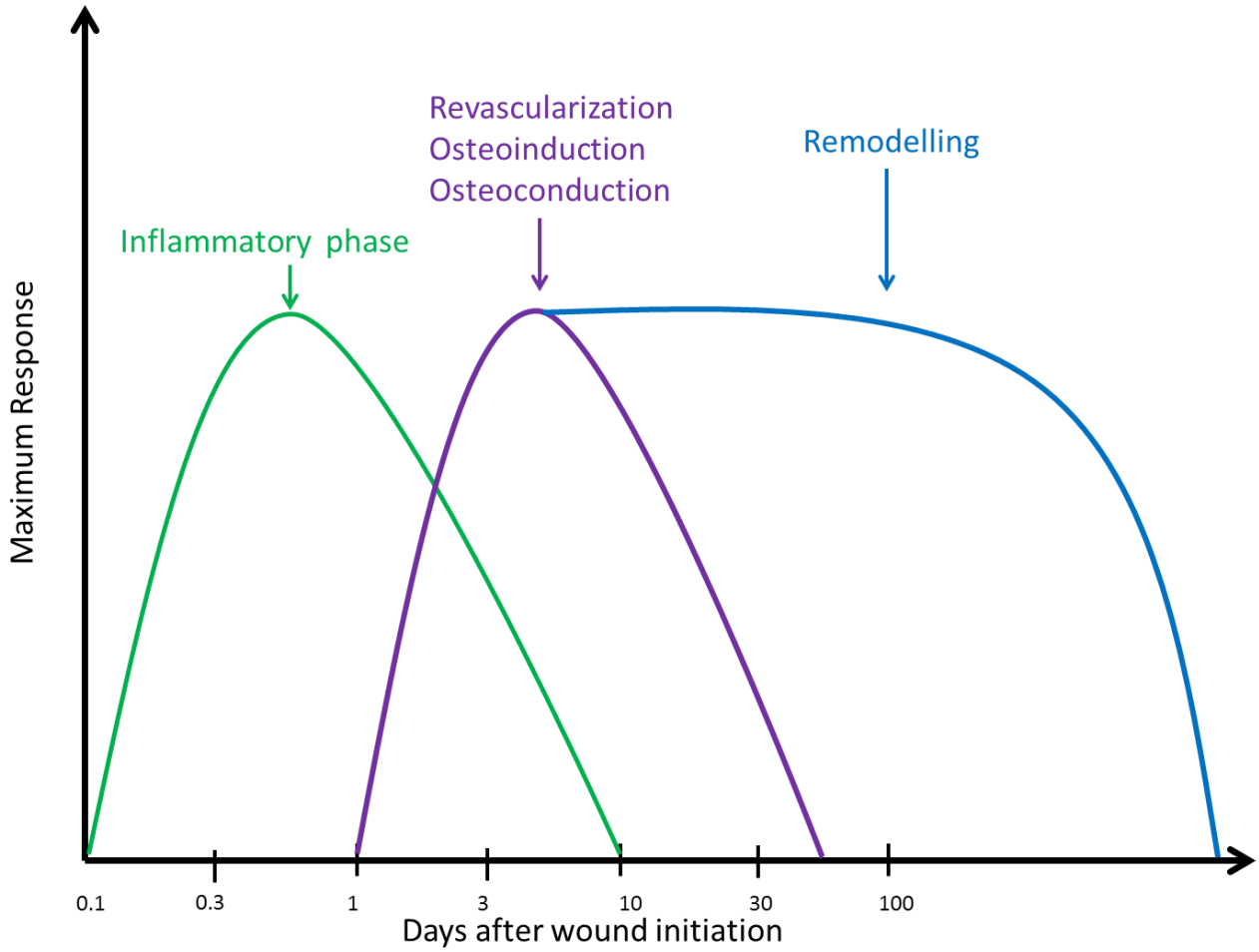


Figure 1: Five phases of autogenous bone graft healing.

1.1.2 Allograft and Xenograft healing

These two distinct transplant alternatives are utilized with similar expectations. It is well known that these bone-derived substitutes harvested from cadavers or xenogeneic sources undergo various treatments before their use in vivo, in order to reduce the host immune reaction.

Preparation of the tissues to be used for grafting may include freezing, freeze-drying, demineralization, or a combination of these processes. Processing methods include physical debridement, treatment with ethylene oxide, antibiotic washing, ultrasonic washing, and gamma irradiation for spore elimination. The required tissue processing of these grafts alters the chemical and functional properties of the bone-graft material, and these processes may affect their remodelling into viable bone. Processing of allogeneic tissues may leave native BMP intact and functional while autoclaving of these tissues destroys BMP activity. Hence, the defatting and deproteinization of allogeneic and xenogeneic tissues required to limit the immune response effectively destroys any osteoinductive properties [29].

Healing of allogeneic and xenogeneic tissues is qualitatively similar to autogenous bone healing, but the process occurs more slowly. Although the initial inflammatory phase of allogeneic and xenogeneic healing mimics that of autograft, the immune response appears to weaken the osteoinductive phase of bone graft integration. While the initial ingrowth of new host vessels may occur quite rapidly in allogeneic tissues, inflammatory cells surround these vessels, occlude them, and lead to their degeneration, with subsequent rapid necrosis of the allograft [8]. Modification of these grafts by freeze-drying or freezing mostly offers a scaffold for the ingrowth of new host bone, and healing occurs via osteoconduction [29]. Osteoconduction may persist for years before remodelling and bone turnover take place.

1.1.3 Alloplastic graft healing

Alloplastic implants have become an integral part of the reconstruction of the maxillofacial skeleton. Some advantages of using alloplasts are their multitude of sizes and shapes, their availability, and no donor site morbidity. Synthetic grafts possess some ideal characteristics including osteoconduction and osseointegration with the native bone. On their own, they do not possess osteogenic or osteoinductive capabilities. Currently, surgeons may have available to them a host of synthetic materials including bioactive glasses, glass ionomers, aluminium oxide, and calcium phosphate. Many alloplasts were developped as rigid implants in order to provide initial mechanical support, and stability to the graft area [29].

Alloplastic materials function similarly to allografts in their osteoconductivity and remodelling properties. Similarly to other osteoconductive agents, alloplasts enable new osteoid formation along its surface, which will later be resorbed during the expected remodelling process. Some alloplasts, such as hydroxyapatite, are more resistant to resorption and are present in the graft site for longer periods. Other alloplasts, such as glass ionomers, are not resorptive at all, and become incorporated in the structure of bone, but never get replaced by host bone.

All alloplastic materials are osteoconductive scaffolds that rely on osteogenesis and osteoinduction at the grafting site in order to provide for bone regeneration. These bioimplants are preferably used in a composite graft where all three ideal bone graft characteristics are met; osteoconduction, osteoinduction, and osteogenesis [3, 8, 32].

1.1.4 Growth factors and bone healing

Growth factors attempt to modify the cellular and molecular interplay that occurs during bone healing in favour of osteogenesis, by directly or indirectly affecting other growth factors, inflammatory cells, and angiogenic factors. As such, these growth factors act as mitogens, by enhancing the proliferation of certain cell types, and as

morphogenetic proteins, by changing the phenotype of the target cells. In addition, growth factors act in both autocrine and paracrine fashion. Elevated serum levels found with certain growth factor use also suggests an endocrine effect. Therefore, growth factors are regulated by other growth factors, enzymes, and binding proteins [33]. They have an effect on multiple cell types thus inducing an array of cellular functions in a variety of tissues [34]. Growth factors also exert their effect on the target cells through surface receptors by activating intracellular phosphorylation enzymes. Following this, a set of genes are activated and exert changes in cellular activity and phenotype [34].

Various hormones and growth factors influence bone repair (table 1). The following growth factors have undergone many investigations for their role in bone healing and angiogenesis: insulin-like growth factor (IGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and various bone morphogenetic proteins (BMP) [34, 35]. Most osteogenic factors stimulate angiogenesis directly, or through the production of angiogenic molecules, such as VEGF[36]. Others influence bone formation through distinct mitogenic effects on osteoblasts. VEGF is known to regulate angiogenesis and stimulate osteoblast differentiation, while BMPs are known to convert mesenchymal stem cells to committed osteoblasts [37]. Similarly, TGF-β is angiogenic and increases the expression of markers of osteoblast differentiation, such as alkaline phosphatase [36, 38, 39]. TGF-β acts as a potent inhibitor of growth for many cells types such as epithelial cells, endothelial cells, hematopoietic cells, and lymphocytes. Its prominent role in bone repair appears to be matrix synthesis of bone cells without playing a role in final differentiation into osteoblasts. Systemic use of TGF-β is limited due to its adverse effects on the immune system [40-43]. Systemic administration of IGF-1 stimulates osteoclast formation and bone resorption, although they have an important role in general growth and maintenance of the body skeleton. BMPs have shown to induce ectopic bone formation in undesirable locations [44], while PDGF has proven to be instrumental in inducing proliferation of undifferentiated mesenchymal cells [33, 45].

While all growth factors are capable of regulating some activity within cells or tissues, it is only BMPs that act as morphogens transforming connective tissue cells into osteoprogenitor cells [6]. The result is that the formation of bone from growth factor

based bone bioimplants is significantly different from autogenic or allogeneic graft healing. Growth factor based bone healing involves promotion of osteogenesis through recapitulation of the developmental process that had originally created that organ or body part during fetal or postnatal growth [33].

In recent years, the use of recombinant DNA technology has led to the identification and molecular cloning of the growth factor BMP [46, 47]. Discovery, purification, and recombinant synthesis of human bone morphogenetic proteins (rhBMP) constitute a major milestone in the understanding of bone physiology [6]. Members of this group of endogenous growth factors have been shown to have osteoinductive activity when implanted into a healthy tissue bed. The term osteoinduction has been utilized to describe the process of " turning on" bone formation, and is a concept that has its roots in the seminal paper by Urist: "*Bone formation by autoinduction*" [48]. RhBMPs provide a limited risk of disease transmission, with reduced patient morbidity, and are a reliable source for the initiation of bone production.

	Osteogenic	Angiogenic	Induction of VEGF
Activins	+	---	TBD
BMP 2, 4, 7	+	+	+
FGF 1-2	+	+	+
GDF 5	---	+	TBD
GH	+	+	TBD
IGF 1	+	+	+
PTH	+	+	+
PDGF	+	+	+
Prostaglandins	+	+	+
TGF-β	+	+	+
VEGF	+	+	---

Table 1: Hormones and growth factors influencing bone repair

1.2 BMP

1.2.1 BMP History and Discovery

As early as 1889, Senn discovered that decalcified bone induced the healing of bone defects in patients being treated for osteomyelitis [49]. In 1938, Levander observed heterotopic cartilage and bone formation when an acid alcohol extract of bone and callus was injected into rat muscle tissue[50]. Shortly after this, in 1945, Lacroix hypothesized the role of osteogenin, an osteogenic inducer of bone [51].

In 1965, Marshall Urist made the key discovery that initiated the hunt for factors responsible for the induction of bone formation [48]. Urist's pioneering work convincingly demonstrated the capacity of devitalized decalcified bone matrix to induce mesenchymal cells from the host to form bone at heterotopic sites. Demineralized bone fragments were implanted subcutaneously or intramuscularly in rats and rabbits. New cartilage and bone appeared at the implantation sites several weeks later [47]. This phenomenon was coined "*The Bone Induction Principle*" by M. Urist, and the protein responsible for this effect was referred to as *Bone Morphogenetic Protein* [34, 48].

Further studies by Urist and Strates in 1971 defined the chemical compounds responsible for bone induction as "*the osteogenic chemical components of the matrix of bone, dentin and other hard tissues that are deinsulated by demineralization and associated intimately with collagen fibrils*" [52]. In 1979, Urist reported isolation of an osteoinductive, water soluble, low-molecular-weight protein from insoluble bone matrix gelatin: bone morphogenetic protein (BMP)[53].

In 1988, Wozney and his coworkers identified the amino acid sequence of BMP, which led to the cloning of its various isoforms [54]. Recombinant DNA technology was later used to clone these genes by inserting them in mammalian and non-mammalian cells. Cellular proliferation led to their large scale production [47]. Currently, 15 different members of BMP have been identified, of which only a subset have osteoinductive capabilities [47, 55]. Therefore, the use of recombinant gene technology has allowed for the generation of a larger, more uniform supply of specific BMPs [56].

1.2.2 BMP Classification

Bone morphogenetic proteins are multifunctional cytokines, and are members of the TGF-β superfamily. The TGF-β superfamily is a large family of growth and differentiation factors including BMPs, TGF-β, and activins and inhibins [55]. There exist several structural homologies between BMPs and TGF growth factors, such as the seven cysteine knot. BMPs have been divided into three groups according to their primary amino acid sequence (figure 2) [55, 57]. Group one consists of BMP-2 and 4, group two consists of BMP-5, 6 and 7, and group three includes BMP-3 [15, 58].

BMP-2 and 4 have 80% homology in their amino acid sequence. In group two, BMP-5, 6 and 7 have 78% homology, whereas in group three, BMP-3 stands alone. BMP-1 is not structurally related to BMPs as it does not retain the amino acid sequence, and thus is not an actual member of the TGF-β superfamily [59, 60].

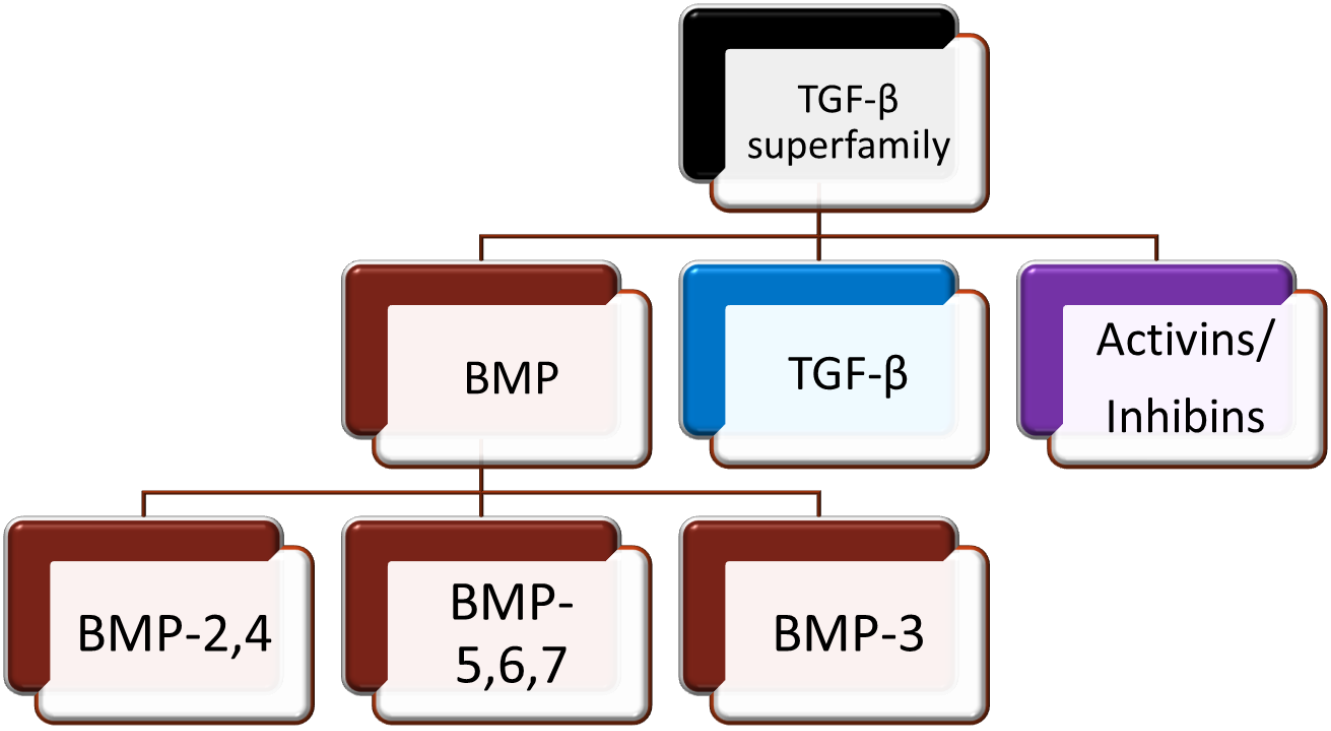


Figure 2: The TGF-β superfamily.

The TGF-β superfamily is subdivided into (1) bone morphogenetic proteins (BMPs), (2) TGF- β, (3) activins/inhibins

1.2.2.1 BMP Receptors

The biologic functions of BMPs are exhibited via specific BMP receptors. There exist two types of receptors, I and II, and both are serine/threonine protein kinases [58]. In mammals, BMP type I (BMPR-I) and type II (BMPR-II) receptors are expressed in several cell lines during embryogenesis [61, 62]. BMPR-II is expressed in skeletal muscle, heart and brain, while BMPR-I is expressed in human foreskin fibroblasts and C2C12 myoblasts, and in brain adult tissue [63-65].

BMPR-II binds BMP-2, 4 and 7 only weakly in the absence of BMP type I receptors [35]. In contrast, BMP type I receptors bind ligands in the absence of BMP type II receptors [66]. However, both BMP type I and II receptors are necessary for the signal transduction for BMPs[35].

1.2.2.2 BMP Signalling

BMPs initiate cell signalling by binding to a transmembrane receptor complex formed by BMP type I and type II serine/threonine kinase receptor proteins. Although specificity in signalling is determined by the BMP type I receptors, transphosphorylation of BMP type I receptors by BMP type II receptors is necessary in order to initiate intracellular signals [67]. It is the binding of BMP to the type II receptors that enables this transphosphorylation to proceed [57, 68]. It is said that type II receptors act upstream of type I receptors, and so one may regard these as primary receptors and transducers, respectively [69].

Once the BMP type I receptors are phosphorylated, they phosphorylate the intracytoplasmic signalling molecules Smad 1, 5 and 8. Hence, the Smad intracellular signalling cascade is initiated. Smads are a family of signalling mediators of BMP receptors. The Smad family includes eight members classified into three groups by structure and function: the signal transducing receptor-regulated Smads (R-Smads 1, 2, 3, 5, 8), the common-mediator Smad (C-Smad, such as Smad-4), and the inhibitory Smads (I-Smads, such as Smad-6 and Smad-7). All Smad proteins, except 6 and 7, share the same structure consisting of the N-terminal and C- terminal domains. R-

Smads are phosphorylated by the activated serine/threonine kinase receptors [35]. Smad 1, 5 and 8, bind to Smad 4 to translocate into the cell nucleus [6]. In contrast, Smad 6 and 7 inhibit BMP signalling. As such, Smad 1, 5 and 8 are involved in BMP signalling, whereas Smad 2 and 3 mediate TGF-β signalling. Once in the cell nucleus, the R-Smad/C-Smad complex binds to the DNA in the regulatory regions of BMP responsive genes. The complex then interacts with RUNX2/CBF, the transcriptional factor that is important in osteoblastic differentiation (figure 3). Transcriptional factor activation regulates the expression of genes involved in cartilage and bone formation. In humans, mutations of the RUNX2 gene may lead to cleidocranial dysplasia [35, 70].

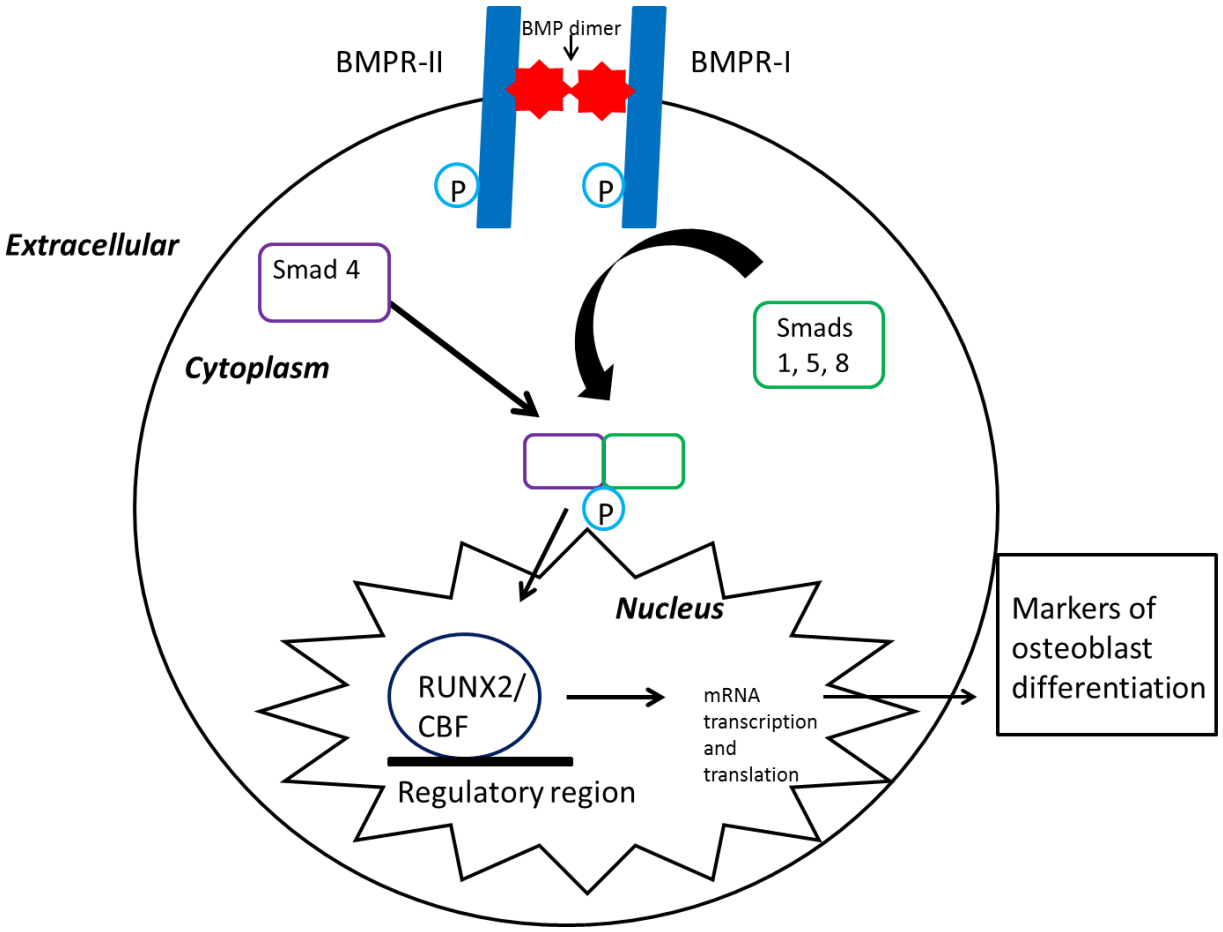


Figure 3: Signaling by bone morphogenetic proteins (BMPs). P = phosphate. Adapted from Hollinger[71]

1.2.3 BMP Structure

As a member of the TGF- β superfamily, bone morphogenetic proteins are low molecular weight disulphide-linked dimeric glycoproteins, and are produced as large precursor proteins that consist of an amino-terminal signal sequence of 15-25 amino acids, a poorly conserved pro-domain of 50-375 amino acids, and a mature carboxy-terminal domain of 100-125 amino acids. The amino-terminal and pro-domain vary in size, whereas the mature domain shows sequence similarity among family members (figure 4)[47]. Prior to secretion from the cell, the precursor molecules undergo dimerization, and proteolytic cleavage separating the mature domain from the pro-domain (figure 5). The active polypeptide is now between 110 and 140 amino acids in length and becomes linked to a second mature region to form the physiologically active BMP dimer.

The mature region of BMPs, now highly conserved, includes seven cysteine amino acid residues. Six of these residues make a rigid structure called a cysteine knot by forming intrachain disulphide bonds. The seventh forms interchain disulphide bonds leading to the production of homodimers and heterodimers [47, 57, 72, 73]. Therefore a BMP consists of a dimeric molecule with two polypeptide chains connected by a disulphide bond. Without this connection, bone induction does not occur (figure 6).

Figure 4: The polypeptide structure of BMP. Adapted from Azari[47]

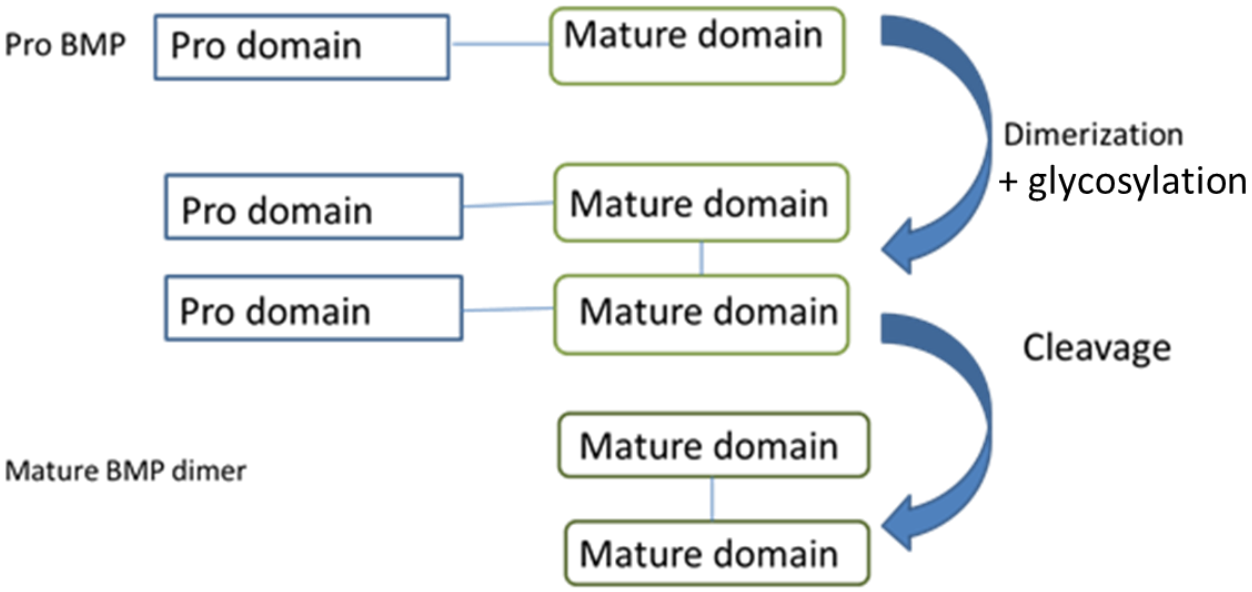


Figure 5: BMP protein dimerization and cleavage to a mature dimeric protein. Adapted from Barr[74]

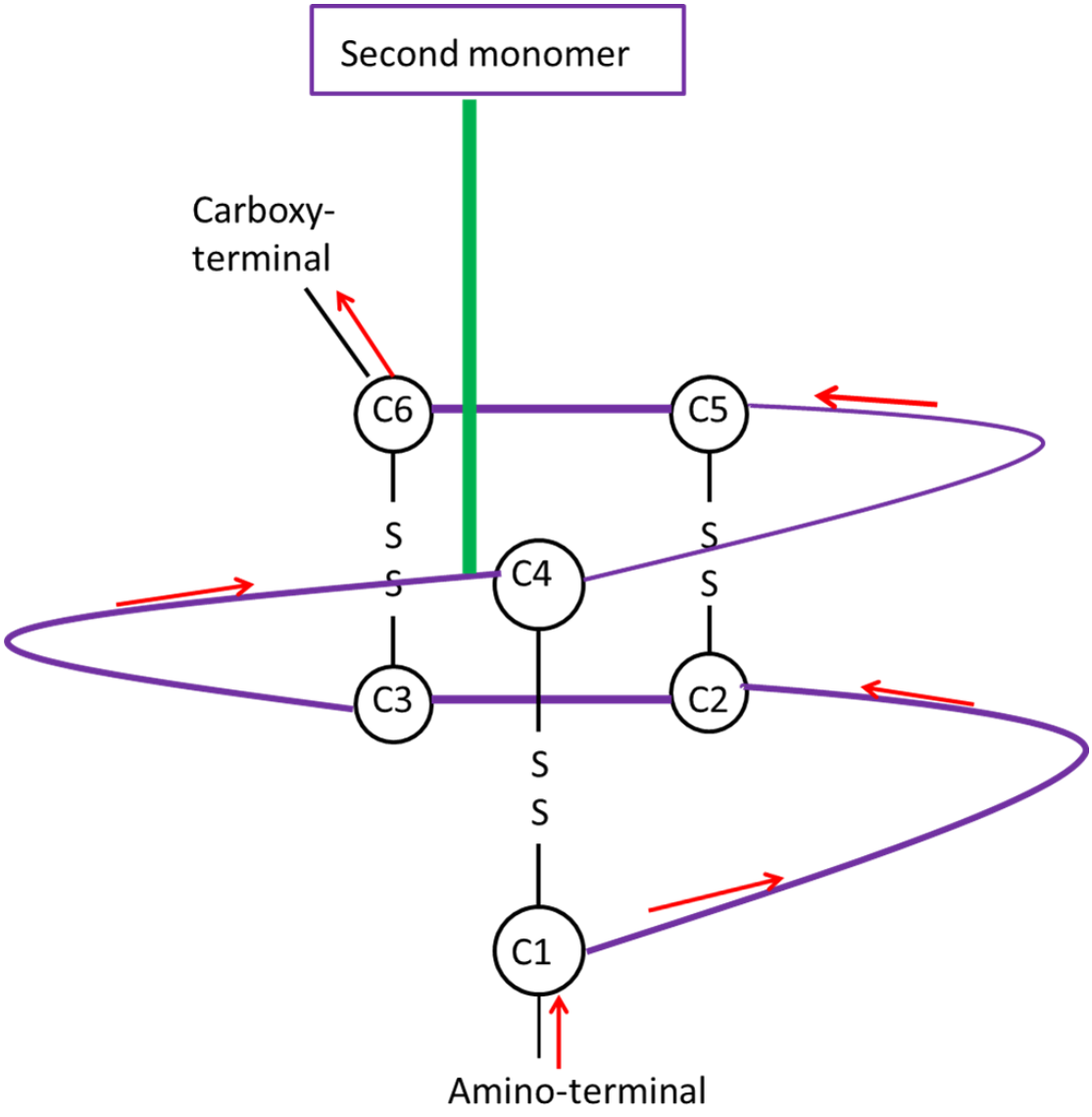


Figure 6: BMP structure: the 7 cysteine knot. C1-6: cysteines. S: sulphide

1.2.3.1 Recombinant Gene Technology and BMPs

Although the native BMP replicates the dosages and ratios in normal bone, interpatient variability and standardization of content are impossible. Recombinant human proteins offer the advantages of accurate dosing, and negligible disease transmission[75]. In addition, the advantages of recombinant production include reproducibility, consistent purity and activity of BMPs, and the availability of large quantities [47, 76, 77].

Human BMP DNA coding sequence is placed in a vector system. This vector is subsequently transfected in a host of choice. In order for the transfected vector to stay in the genome of the host cell, stable transfection must occur. To achieve stable transfection, a resistance gene is co-transfected which provides the host cell some selectable advantage. The host cells with the resistance gene integrated into their genomes will be able to proliferate, while other host cells die when exposed to a selection agent. After selection pressure, the host cells with a stable transfection replicate. These cells are screened to assure secretion of BMP. This leads to large scale BMP protein synthesis. BMP-expressing cells are then purified, and the BMP molecules are dimerized, processed and glycosylated as are the naturally occurring molecules. The mature active protein is a 30kDa homodimer that resembles natural bovine bone [78] (figure 7).

To date, a number of preclinical studies have assessed the efficacy of recombinant human BMPs in the healing of critical-sized bone defects in rats, rabbit, sheep and dog models[79-81]. Hollinger et al defined a critical size defect as "*the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal*" [82, 83]. Two rhBMP products are currently available for clinical applications: recombinant human BMP-2 (Infuse) and recombinant human BMP-7 (osteogenic protein 1-OP1). The activity and efficacy of recombinant BMP-2 requires more research to more fully apply it to clinical practice.

After ectopic implantation of rhBMP, a sequence of events typical to endochondral ossification follows, with the five distinct phases of healing; recruitment of mesenchymal cells and differentiation to chondrocytes, chondrocyte hypertrophy, calcification of

cartilage to matrix, osteoblast differentiation and bone formation, and remodelling of newly formed bone[84].

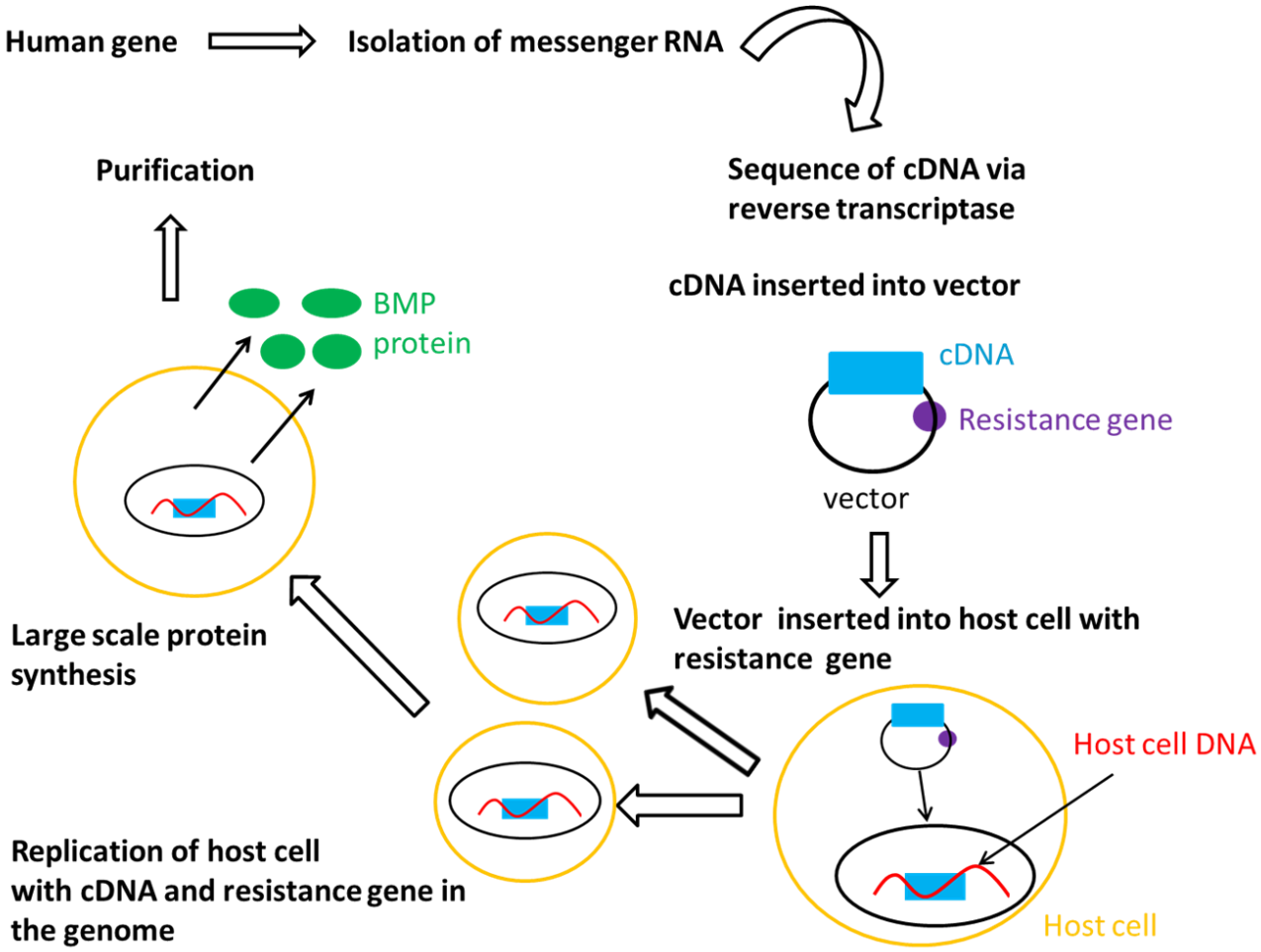


Figure 7: Recombinant gene therapy. Adapted from Lindholm [85]

1.2.4 Physiological Roles of BMPs

BMPs are multifunctional proteins exhibiting a wide range of biological activities through the signalling of various cell types, including epithelial cells, mesenchymal cells, neuronal cells, and monocytes. As a result, BMPs have an important regulatory role in many aspects of embryologic development, and craniofacial and limb development. Extracellular matrix comprises the main source of BMPs produced by osteoprogenitor cells, mesenchymal cells, osteoblasts and chondrocytes. In vivo, BMPs have been found to be expressed in cells in developing bone, in fracture callus, and in ectopic bone. They may also be expressed in other tissues such as hair follicles, heart, kidney, tooth buds, oocytes, prostate, and central nervous system [86, 87].

1.2.4.1 Role of BMPs in Embryogenesis

Recombinant BMPs are effective in lower life forms, like fruit flies, because the amino acid sequence is highly conserved, and is considered to be as old as 600 million years [6]. There exists 75% sequence homology between BMP-2 and Drosophila decapentaplegic protein (dpp), a protein involved in dorsal-ventral patterning, gut morphogenesis and wing vein formation during embryogenesis of fruit flies. Studies have shown that dpp induces bone and cartilage in mammals, which leads to believe that dpp and mammalian BMPs are interchangeable. Hence, BMP plays a pivotal role at the very early stages in embryogenesis, as it is essential in dorso-ventral patterning of the mesoderm layer. Studies on these other species have provided valuable information for understanding the roles of BMP in mammals [35, 63, 88].

Further studies have elucidated the role of BMPs during early embryogenesis, organogenesis and skeletogenesis. For example, short ear mutations in mice are associated with deletions of the BMP-5 gene [89]. While BMP-2, 6 and 9 appear to be the most potent inducers of osteoblast differentiation of mesenchymal progenitors cells, BMP-2 deficient mice had amnion-chorion malformation with subsequent cardiac defects [15, 90]. Furthermore, death in mice with null mutation in the BMP-7 gene ensues shortly after birth, with significant defects in kidneys and eyes morphogenesis, showing the role of BMP-2 in organogenesis [35, 91]. Also, BMP-2 and 4 knock-out mice die early in embryonic development, long before development of the skeleton,

revealing the importance of BMP in skeletogenesis [39, 92]. Therefore BMP family members are expressed primarily during embryologic development, endochondral ossification, and early fracture and cartilage repair [76].

1.2.4.2 Role of rhBMPs in Bone Repair

RhBMP application has been used in several clinical situations including, non-union fracture healing, spine fusion, healing in infected bone, and osseointegration of orthopedic implants [93, 94]. Preclinical and clinical research has been performed to examine the feasibility, the safety and the efficacy of rhBMP/ACS in the treatment of common oral and maxillofacial defects. Published data suggests that rhBMP combined with an absorbable collagen sponge carrier can induce new bone formation, and heal critical-sized defects in extremities and in the maxillofacial complex in animals. As preclinical studies did not reveal major adverse effects, BMP clinical trials were initiated [84, 95, 96].

In a clinical study by Fiorellini et al in 2005, two doses of rhBMP-2/ACS were examined in 80 patients requiring extraction socket augmentation. Their results demonstrated that 1.5mg/cc rhBMP-2/ACS treated sites had double the amount of bone compared to the empty control group [97]. Similarly, a two phase study by Boyne in 1997 and 2005, also identified 1.5mg/cc of rhBMP-2/ACS as the most effective dose in evaluation the feasibility in maxillary sinus floor augmentation [98, 99]. These clinical studies demonstrated that the bone induced by rhBMP-2/ACS was found to be biologically similar to native bone, but that the higher concentration of rhBMP-2 induced bone formation at a faster rate than the lower concentration [100, 101]. RhBMP-2/ACS is therefore adequate to support the functional loading of dental prostheses [97, 98, 102].

Many similar studies support the use of rhBMP/ACS in maxillary sinus floor augmentation, alveolar ridge preservation and implant osseointegration [57, 97-100, 102, 103] . A study by Sigurdsson, and another by Hanisch, both showed that rhBMP/ACS is successful in augmenting bone with and without guided bone generation membranes when used as an inlay to treat space-preserving defects. As such, intrabony and saddle-type defects are more amenable to treatment with rhBMP, where compression is not a problem, versus critical size defects where compressive resistance

is necessary [104, 105]. Reconstruction and osseous regeneration of large traumatic, congenital and pathological-sized defects of the facial skeleton continue to present a challenge for the treating craniofacial surgeon. Studies in large nonhuman primates were conducted in order to better document the ability of rhBMP-2 to induce bone formation in critical-sized defects. In all of these studies, histological and radiographic assessments revealed progressive ossification of intramembranous nature [106-108].

Another area of clinical interest is congenital cleft lip and palate. Currently, alveolar clefts are usually treated with autogenous grafting. The use of 1.5mg/ml of rhBMP-2/ACS in 50 typical unilateral or bilateral clefts has demonstrated successful union of clefts with consolidated bone in the alveolar cleft and eruption of teeth in the reconstructed area [109, 110]. Although these studies suggest superior results with rhBMP rather than autogenous bone in adult alveolar clefts, FDA approval has not been granted for the use of rhBMP in the pediatric population. The question remains whether there may be any short or long-term effects on the growing bones and neural development [111].

1.2.5 Safety of rhBMP

Purity, localized activity, systemic availability, immunogenicity and biocompatibility all have an impact on the safety of rhBMP. As tightly folded, disulphide bond structures, BMPs are intrinsically stable proteins[4]. RhBMP-2 has been extensively studied for its potential toxicity, immune reactivity and uncontrolled bone formation[112]. Reports of low titers of antibody formation to type 1 collagen have been described in clinical spinal fusion trials with rhBMP /ACS, however, no harmful outcomes were reported [113].

In 2002, a report suggested that the use of supraphysiologic doses of rhBMP did not show harmful systemic or toxic effects [114]. Others have suggested that restenosis can take place if rhBMP contacts raw surfaces, such as laminectomy sites [115].

Long-term concerns about the use of rhBMP are still unknown, such as the effect of high doses of BMP on a developing embryo, and as such, should not be used in

pregnant women [6, 112]. Also, the use of rhBMP-2 is contraindicated in patients with hypersensitivity to rhBMP-2 or bovine type 1 collagen. In addition, rhBMP-2 should not be used in patients with active malignancies, in areas of active infection, and in areas of tumour resection [112, 116].

Recent studies in the realm of adult spine surgery questioned the side effects of synthetic BMPs. Complications have been reported including renal or hepatic failure, wound complications, compartment syndrome, severe edematous swelling, heterotopic bone formation, and carcinogenesis[117, 118].

1.2.6 Source of rhBMP-2

In selecting a suitable host cell for protein production, many considerations are to be followed such as the ability to perform post-translational modifications, the ability to affect extracellular expression, folding, and the biological activity of the protein of interest. Economic issues in the large-scale production of the protein must also be considered [119]. Glycoproteins are important in human therapy where pharmacokinetic properties and receptor targeting are dependent on the presence of specific sugars on the protein [120-122]. Oligosaccharide structures on glycoproteins ensure solubility and prevent aggregation [120]. The solubility of rhBMP-2 is greatly dependent on its glycosylation [123]. Resistance to protease attack reveals stability of a given glycoprotein[124]. As such, glycosylation modulates protein solubility, folding, secretion, thermostability, catalytic efficiency, antigenicity, recognition and clearance [125].

RhBMP-2 may differ slightly from their bovine counterpart in that post-translational processing can lead to aberrant proteolytic cleavage. This could affect the specific activity of the rhBMP-2 by reducing its affinity for the receptor. Finally, activity can be affected by incomplete processing of rhBMP-2, and the purity of the implanted samples [126, 127]. Despite the slight differences between non-recombinant BMP and rhBMP, the overall structure and activity remains very similar.

There exist many different types of host cells for the production of recombinant proteins: mammalian cells, bacteria, fungi, yeast, plant tissue culture, transgenic animals, and transgenic plants [128-130]. The most common host cell line used in biotechnology is the Chinese hamster ovary cell (CHO). RhBMP-2 protein from CHO cells highly resembles native BMP in structure and activity. However, the osteoinductive properties of recombinant BMPs from CHO cells are slightly reduced compared to their purified bovine counterpart [127]. Mammalian CHO cells have the ability to carry out proper protein folding of rhBMP, and N-linked glycosylation, thus eliminating the need for renaturation [131, 132]. Their extensive post-translational modification machinery gives them the ability to produce mature proteins through proteolytic processing and leads to efficient secretion of the protein from the cell into the culture medium [133]. The processed forms of rhBMP-2 from mammalian CHO cells highly resemble native bovine BMP: similarly to non-recombinant BMP-2, rhBMP-2 must undergo proteolytic cleavage to remove it from the mature domain prior to the secretion from the cell in order to become active [133].

It has been suggested that mammalian cell processes have the potential for product contamination by viruses [128]. In addition, the system is not ideal for preparing a large quantity of rhBMP-2 [134, 135]. With BMP gene-transfected mammalian cell cultures, cultivation is expensive and yields are poor [54, 57, 78].

Researchers have therefore turned their attention to the production of rhBMPs in the prokaryotic host, where, theoretically, yields are high, costs are low, and bio- safety is favorable [127]. As part of the prokaryotic system, E-coli is believed to be favorable as a simple, rapid and relatively inexpensive system. However, it is not the system of choice for disulfide rich proteins, and proteins that require post-translational modifications. Furthermore, for proteins requiring glycosylation, mammalian cells, fungi and baculovirus systems are preferable for biological activity[128]. RhBMP-2 from E-coli cells form inclusion bodies that are structural dissimilar to native BMP, and require extensive post-translational modifications to achieve the active form. Furthermore, the refolding and renaturation procedures are complicated which can lead to overall low yields [136, 137]. The production of biologically active rhBMP-2, through in vitro refolding of Escherichia coli produced inclusion bodies, has been attempted in order to

provide large quantities of rhBMP at a lower cost for clinical therapy. Although inclusion bodies have a number of advantages such as high levels of enriched protein production, protection from proteolytic degradation and easy purification, they require solubilization and renaturation procedures that may be inefficient, rendering them biologically inactive. In contrast to CHO BMP protein, bacterial BMP protein is not protected from intracellular proteolytic degradation during protein secretion and inherently lacks the eukaryotic N-glycans [125]. The reducing environment of the cytoplasm prevents the formation of the stable disulfide bonds, therefor disfavoring the correct folding of complex proteins, and favoring the formation of inclusion bodies [120, 126, 134, 138-140].

In order to obtain an active protein, the inclusion bodies are first removed from the cell, the proteins are solubilized by denaturants which unfolds the proteins, and disulfide bonds are eliminated using reducing agents. Following this, refolding is achieved by removing the denaturant and the reducing agent. Renaturation then occurs through processes including air oxidation, glutathione reoxidation system, and mixed disulfided of protein –S-sulfonate and protein –S-glutathione system [141]. Several factors affect the degree of protein folding and aggregation during the refolding process: protein concentration, detergents, temperature, pH, redox environment, ionic strength, and polymers. A low overall yield has been reported for rhBMP-2 production through the in-vitro refolding [136].

As a result, there has been extensive research to develop a simple and inexpensive method to improve the production of biologically active rhBMP-2 from bacterial cell cultures that are structurally different from the natural BMP [134, 140, 142-145].

In order to consider rhBMP-2 prepared from a prokaryotic microorganism as an economic alternative to CHO cell rhBMP-2, the osteogenic activity of both rhBMP-2 has been compared [146, 147]. Despite its lack of glycosylation and complicated refolding procedure, E-coli rhBMP-2 revealed promising osteogenic capacity at a level equivalent to that of CHO cell rhBMP-2 [146-149]. Hence, E-coli rhBMP-2 may be considered a valued alternative in BMP therapy (table 2)

rhBMP Source	
CHO	*E-coli*
Proper folding Glycosylated Disulfide bonds	Inclusion bodies Non glycosylated No disulfide bonds
Expensive Low yields	Low costs High yields

Table 2: rhBMP source: CHO versus E-coli features

1.2.7 Carrier Systems for rhBMPs

The effectiveness of rhBMP is dependent upon both the intrinsic biological activity of the protein, and the method used to apply that protein[4]. BMPs are locally acting differentiation factors, highly soluble, and cleared rapidly if delivered in a buffer solvent. By reason of their sensitivity to endogenous proteases, rhBMPs, like other growth factors, have to either be protected or delivered by carriers at a continuous rate at the site of interest. In addition, protein carriers are used in order to maintain the concentration of rhBMP at the repair site for longer period of time to fully benefit from its inductive properties. The choice of delivery system is primarily influenced by the size and the nature of the defect to be reconstructed. They localize rhBMP to the defect site, allowing slow release of the protein which further helps prevent systemic toxicity. In some cases, such carriers may act as osteoconductive matrices[79].

The carriers most frequently tested for rhBMP include synthetic polymers, natural polymers, and inorganic materials.

Synthetic polymers eliminate the possibility of disease transmission, offer a predictable resorption, and can be supplied without limits. They are typically processed into highly porous scaffolds. Commonly used synthetic polymers include poly α-hydroxy acids, such as polylactide, and polyglycolide [150]. The breakdown products of these materials have been proven to elicit a foreign body giant cell reaction and chronic inflammation, making them poorly suited for bone regeneration [77, 151].

Natural polymers include collagen, fibrin, alginate and plant-derived polysaccharides. Collagen is the major non-mineral content of bone. Collagen formulations include gelatin, demineralized bone matrix (DBM), and fibrillar collagen, of which most are composed of type 1 collagen derived from porcine bone, skin or tendons[152]. DBM contains non-collagenous proteins including endogenous osteogenic factors. While being one of the first collagen-based carriers used in rhBMP delivery, DBM presented issues relating to immunogenicity, risk of disease transmission, and problems with maintenance of fine particles which has pushed the development of injectable gels, and cross-linked sponges [152]. Currently, the collagen sponge is used as the primary delivery method for rhBMP-2. The commercially available absorbable collagen sponge

(ACS) is fabricated from bovine tendon-derived collagen. The manufacturing of the collagen sponge begins with processing the purified collagen material into an aqueous solution. In order to manufacture a collagen device with a homogenous texture, freeze-drying or lyophilization is the best process [153]. Lyophilization of a dispersion of bovine Achilles tendon collagen is performed, followed by crosslinking and chemical sterilization [154, 155]. Crosslinking is performed if the three dimensional structure of the collagen is not holding in the presence of a liquid [156]. Sterilization of collagen with steam or chloroform is not advisable because of the irreversible damage to the helices [157]. Therefore, gamma and electron beam irradiation are used as alternate methods of sterilizing collagen matrices. As a carrier, collagen has the advantages of being biodegradable and biocompatible [158, 159]. It binds rhBMP and other growth factors to present them to the responding osteoprogenitor cells, such as mesenchymal cells, for bone regeneration [85, 160]. Yet, ACS cannot be molded to a desirable shape for bone augmentation [161]. It lacks inherent strength, and therefore does not have the ability to maintain space during bone induction[112]. In addition, the more rapid release of rhBMP-2 from a collagen sponge has the potential to stimulate a more aggressive response, which is biologically undesirable [146]. Hence, ACS has its limitations as a carrier for rhBMP-2 use.

The category of inorganic materials includes calcium phosphate ceramics, such as hydroxyapatite (HA) and tricalcium phosphates (TCP), their mixture which is called biphasic calcium phosphate (BCP), and non-ceramics, such as calcium phosphate-based cements (CPC). These materials differentiate themselves from one another and from bone in composition and physical properties [162, 163]. Certain calcium phosphate ceramics have the ability to attract and concentrate endogenous BMPs therefore enhancing osteoinductivity [162]. These actions prolong the exposure of the protein to stem cells, and increase the duration of interaction between proteins and other growth factors, leading to a synergistic activity [164]. Hydroxyapatite (HA) has been widely used as an osteoconductive carrier matrix because the primary mineral component of bone is HA, and osteoblasts deposit easily on this material [165]. Supplied in granules formulation, these ceramics offer the added advantage of increased surface area for rapid bone stem cell penetration and vascular invasion. A

high surface suggests a higher propensity for binding BMP. Although HA shows excellent biocompatibility, and possesses sufficient strength to withstand compressive forces, it is resorbed slowly, and prone to cause infection and excessive tissue reaction as a foreign body, making this material less than ideal for protein carrier use [165-168]. Tricalcium phosphate (TCP) granules share many of the same characteristics as HA. They have good biocompatibility, and bind to bone [82]. However, TCP granules are more biodegradable than HA, and they resorb up to 80% after 6 months [82].

In addition to prolonging the retention of BMPs at the site of implantation, TCP granules offer a slow release of rhBMP-2, therefore reducing the negative effects of an aggressive response and allowing prolonged sustained release of the osteogenic protein. Like HA, TCP can be brittle and has low impact resistance. In addition, it has been noted that macroporous TCP alone will undergo moderately rapid degradation which is completed by nine months, with evidence of fibrous tissue in the pores [82, 169]. Because HA can remain at the site for years, and the TCP has the potential to resorb before bone formation may occur, BCP (biphasic calcium phosphates) bioceramics are a combination of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) in varying ratios, that have been developed [32, 170]. The advantage of the BCP over other calcium phosphate ceramics is its ability to gradually dissolve in the biological medium therefore releasing calcium and phosphate ions leading to new bone formation [171]. Its main attractive feature as a bioactive bone graft material is its ability to form strong bonds with the host bone which leads to a robust interface, versus a fibrous interface [162, 172].

Injectable calcium phosphate cements harden in vivo and have proven to create robust bone. One disadvantage of the calcium phosphate based cements is its poor mechanical properties, which has limited its use to low-stress bearing applications [173].

The combination of these materials optimizes the benefits offered by each material. It allows for a more ideal carrier matrix with the controlled release of synthetic polymers, the biocompatibility of natural polymers, and the osteoconductive potential of bioceramics [174].

An ideal carrier should have the following characteristics: biocompatible, biodegradable, not immunogenic, clinically accessible, able to retain incorporated molecules, affinity to rhBMP, amenable to sterilization, stable against compression and tension, resorbable at a rate similar to the neighbouring bone product, inexpensive and readily available[175]. It is generally agreed that the pore size should be comparable with that of cancellous bone [6]. Matrices must be protective of rhBMPs from non-specific lysis, and promote rapid vascular invasion if residence time will be increased [79]. The rate of degradation of the carrier must be compatible with the rate of new bone formation in order not to compromise mechanical integrity of the repair. Longer retention ultimately results in higher osteoinductive activity [174]. Factors affecting the retention of the rhBMP protein to the carrier may include the carrier geometry, the affinity of the protein to the carrier, the mechanism of protein release, and the carrier degradation.

1.2.7.1 Carrier geometry

Carrier porosity and interconnectedness of pore, pore size, carrier volume and surface area dictate the diffusion distance. Denser collagen sponges have more binding sites, and thus less unbound rhBMP-2. Incorporation represents the rhBMP-2 absorbed to the collagen sponge plus the protein dissolved in the liquid that cannot be removed by rigorous squeezing [176]. Crosslinking of the collagen reduces rhBMP-2 incorporation by direct physical hindrance, thus reducing binding sites. However, in vivo, it leads to prolonged rhBMP-2 residence time and $t_{½}$ [177]. $T_{½}$ is defined as " the time required for a living tissue, organ, or organism to eliminate one-half of a radioactive substance which has been introduced into it" [178].

The surface and chemical characteristics of the HA component of the BCP has shown to have an important effect on differentiation of bone. Two features of the carrier material are necessary to induce ectopic bone: micro and macroporous structure [179, 180]. Macroporous HA, with its optimal interconnected pore structure, has favored capillary invasion through the pores, thus increasing the oxygen delivery to osteogenic cell from perivascular mesenchymal cells, ultimately enabling bone formation [181, 182]. It is suggested that surface area is increased with the micropores such that cell adhesion of the material is enhanced [180]. In 1998, Yuan suggested that porous

ceramic blocks are favorable to ectopic bone formation, and that material of granular form has shown to form bone trabeculae in between the BCP particles. He reported that the amount of bone formation was dependent on the particle size, the optimal size being 250um [180]. Hence, the granular structure of the BCP carrier may be conducive to bone formation through increased space preservation and surface area.

1.2.7.2 Affinity of the protein to the carrier

The affinity of the protein to the carrier is dictated by the specific chemical binding groups between the protein and the carrier. For example, cells from the collagen sponge have specific cell-surface receptors that interact with both ECM molecules and BMPs. Many factors involved in the preparation of the collagen sponge may affect its performance [176]. For instance, the sponge mass, crosslinking, and sterilization have a direct impact on the interaction of rhBMP-2 and the carrier, and on in vivo retention of rhBMP-2. Moreover, implant soak time, protein concentration, pH and composition of the buffer, and rhBMP-2 affect total protein load, and in vivo retention [176].

Typically, the rhBMP protein is provided as a lyophilized powder that is then dissolved into solution. The solution of lyophilized protein is then mixed with a lyophilized collagen sponge, or calcium phosphate granules. Many authors have found that by increasing the soaking time, protein incorporation is increased [154, 176, 183]. In the interest of maximizing binding of rhBMP-2 to ACS and avoiding rhBMP-2 precipitation, it is important to control pH, anion concentration, crosslinking and ACS mass [183].

Similarly, the reactivity of the CaP is dependent on the method of formulation of the BCP carrier (precipitation, hydrolysis, or mechanical mixture), the pH, and the temperature. Temperatures above 700 ^{0}C are used for sintering of the synthetic calcium apatite in order to obtain BCP product [172, 184, 185].

Therefore, preparation of the carrier plays an important role in the affinity of the protein to it, ultimately affecting the biological availability of the protein.

1.2.7.3 Mechanism of protein release

The mechanism of release is highly dependent on the carrier choice and the method of loading the protein onto the carrier. Two major mechanisms of protein release exist: biodegradation of the carrier or diffusion through the carrier. RhBMP release kinetics are also dependent on the animals species. For example, rodents are faster healing species and tolerate a much quicker release profile than metabolically slower species, such as humans [186].

Bolus releases lead to rapid diffusion of rhBMP-2 away from the site of interest, thereby necessitating supra-physiological doses of the protein in order to achieve a critical density of osteogenic infiltrative cells. Clinically, these doses have the potential to create ectopic bone formation, and severe inflammation [187-189]. In addition, the rapid release of the protein may result in transient osteoclast-mediated resorption of newly formed bone [139, 190, 191]. On the other hand, slow releasing systems may never attain the threshold level of rhBMP necessary to trigger local and distant cell infiltration required for bone induction. Hence, extremes of release are not recommended for bone induction [186].

It has been proposed that the preferred kinetics for rhBMP is an initial burst to first recruit stem cells to the implantation site, followed by a sustained release($t_{1/2}$ 3-5 days) to promote vasculogenesis and parallel the differentiation of osteoprogenitor cells to osteoblasts[186, 192]. The initial burst release is said to be carrier independent, in contrast to the secondary release, which is carrier dependent [77].

In vitro studies have revealed that soak-loaded carriers, such as ACS, have an initial burst with release of up to 90% of rhBMP-2 from the ACS within the first 24-48 hours, followed by a prolonged release until day14 [186, 193]. In vivo studies have found that the rhBMP-2 release from the collagen sponge is more sustained than in vitro. The gradual loss of rhBMP-2 after the initial burst has revealed to parallel the gradual degradation of the collagen[152].

Some calcium phosphate materials appear to have an irreversible binding of a fraction of the rhBMP-2 during the secondary release phase. The mineral carrier does not

resorb as readily as the collagen sponge. RhBMP-2 also coats the surface of the calcium phosphate granules [194]. In bone repair models, porous and granular calcium phosphate materials have led to higher efficacy compared to more solid materials [77, 195-197]. It is the difference in binding affinity and in surface area among these carriers that explain the difference in extended release profile in vivo [198].

This means that scaffolds that enable an initial burst followed by a sustained release of the protein promote significantly more bone formation than those with sustained release only [199]. In this regard, ACS may be favorable compared to BCP as a carrier.

1.2.7.4 Carrier degradation

Degradation of the carrier matrix should ideally be in synch with the rate of osteogenesis induced by the protein. Biodegradation can be influenced by the experimental model, implantation site, and animal species [172, 200]. Slowly degrading matrices may inhibit the naturally occurring regenerative process and inhibit bone remodelling. These matrices may become encapsulated by a bony shell, thereby becoming isolated from the repair process. This effect is more apparent with slow degrading granules, such as hydroxyapatite [186]. However, a fast degrading carrier may prevent the filling of the defect which uses the scaffold as a guide. This can lead to incomplete filling of defects, with ingrowth of unwanted tissue. Sciadini et al in 2000 evaluated the efficacy of rhBMP-2, delivered in a collagen sponge, in the healing of a critical-sized radial defect in a dog model. They found that a dose-dependent occurrence of cyst-like voids in the bone was apparent both radiographically and histologically [81]. They hypothesized that rapidly resorbable carriers, such as collagen sponge, may leave voids not filled with new bone.

Collagen sponges are degraded by enzymatic processes; absorbable collagen sponge can degrade within 2 weeks [198]. Calcium phosphate granules are degraded either via cell-mediated processes or liquid dissolution [186, 197]. Dissolution of biphasic CaP includes the degradation of the HA and TCP crystals. In vivo dissolution is dependent on material ratio in that the higher the ratio of β-TCP/HA, the greater the resorbability [201].

The collagen sponge and calcium phosphate granules' breakdown products lead to osteoconductive matrices [197].

None of the current carriers meet the requirements for the ideal carrier system [75]. This has resulted in the use of high doses of rhBMP, which can lead to the formation of voids in the repair tissue. Ultimately, high doses of BMP may lead to osteoclastic resorption before osteoblastic appearance, resulting in loss of strength [81]. It has yet to be determined which carrier is more favorable to bone formation: ACS or BCP (table 3)

	ACS	BCP
	Biodegradable Biocompatible	Biodegradable Biocompatible
Affinity of BMP to the carrier	↑soak time = ↑BMP incorporation ↑pH = ↑ BMP retention BMP carrier	Ionic charges Endogenous BMP concentrators
Carrier geometry	↑Crosslinking = ↓BMP incorporation Weak structural integrity	↑granular surface area + porous structure= ↑ BMP binding sites Space preservation
Protein release	Initial burst release/diffusion through the carrier	Slow release/biodegradation of the carrier
Carrier degradation	Enzymatic process Fast	Cell-mediated process/liquid dissolution Slow-release of Ca and PO_4

Table 3: ACS versus BCP properties

1.2.8 Dose of rhBMP

It is believed that the doses of rhBMP required for bone induction in humans is considerably higher than endogenous BMP concentrations [84, 202-204]. Some hypothesize that exogenously delivered rhBMPs turn over rapidly in acute wounds, leading to the necessity of the delivery of high doses [205]. Therefore, their belief is that supra-physiological doses of rhBMPs are required to induce bone [205, 206]. Higher concentrations would also be required to overcome the tight regulation of these factors and their inhibitors [122, 207-209]. Also, vascular sites and sites with high fluid content may necessitate higher doses to counteract the higher rhBMP clearance rate[186]. The manufacturing costs of such high doses may present a financial problem for the patient, in addition to the potential biological risks associated with such doses [57].

Various factors may ultimately affect dose dependency such as the function of the delivery system, the host species, and the experimental site [147]. Several studies have looked at the effect of dose of rhBMP on bone formation [78, 81, 146-149, 210, 211]. Low doses of rhBMP result in little cartilage, while high concentrations result in direct (intramembranous) ossification [4, 84, 202].

Many studies have revealed promising activity of E-coli rhBMP-2 on the collagen sponge in vivo, and considered the E-coli derived rhBMP-2 as an alternative to rhBMP-2 in CHO cells for clinical use[147-149]. For example, in 1998, Kubler et al demonstrated that E-coli derived rhBMP-2 was as active as CHO derived rhBMP-2 at equal concentrations and in different assay systems [149]. The in vivo experiments consisted of using different concentrations (0.4, 4, 40ug) of rhBMP-2 bound to a collagen carrier and implanting it intramuscularly in the abdominal wall of rats for 28 days. Samples with a concentration of 4ug of rhBMP-2 showed induced cartilage tissue at 28 days, whereas samples with 40ug of rhBMP-2 showed extensive bone formation at 28 days. In 2010, Lee et al investigated the ability of rhBMP-2, on an absorbable collagen sponge to form ectopic and orthotopic bone in rat models. There was no direct comparison to a mammalian system, but the dosage and method highly resembled those in this current investigation. At rhBMP concentrations of 2.5, 5, 10, and 20ug, E-coli derived rhBMP-2 was loaded on an absorbable collagen sponge and implanted either in a rat calvarial

defect or in a subcutaneous back pouch, and analysed at 2 and 8 weeks. In the ectopic model, results revealed bone formation as early as 2 weeks, with woven bone at the periphery of the implant and loose connective tissue at the core. At 8 weeks, histological observation revealed advanced remodelling. The rat subcutaneous pouch model resembles the mouse muscle pouch model used in this study [147]. By comparing their results to the literature, these authors found that the specific activity of the E-coli derived rhBMP-2 was similar to that of recombinant BMP-2 in CHO cells, and that E-coli derived rhBMP-2 can be considered an alternative to rhBMP-2 in CHO cells for clinical use in humans [54, 127, 147, 148].

Results are conflicting regarding equivalent osteogenic potential between the E-coli rhBMP-2 and CHO rhBMP-2. Studies lack in proving significantly higher osteogenic potential at equivalent doses between E-coli derived BMP and CHO derived BMP.

1.2.9 Summary

Two commercially available rhBMPs have been approved for clinical use: rhBMP-2, and rhBMP-7. RhBMPs are most commonly produced in chinese hamster ovary cells or E-coli.

There are two commercial available rhBMP-2 based bioimplants for human application: Medtronic's mammalian Infuse bioimplant, and CowellMedi's E-coli bioimplant Co., Ltd,

The objective of this study was to pursue a direct comparison of mammalian derived rhBMP-2 (Induce Biologics and Medtronic's Infuse Bioimplant) containing CHO rhBMP-2 combined with absorbable collagen sponge, to E-coli derived rhBMP-2 (CowellMedi) containing E-coli produced rhBMP-2 combined with 70% tricalcium phosphate (TCP)/30%hydroxyapatite (HA) granules, at doses stated in the literature to be most effective, in order to elucidate the magnitude of their osteoinductive activity, and evaluate the effect of the source and the carrier on overall bone activity.

2 Materials and Methods

2.1 Research Design

The aim of this study was to compare the osteoinductive capacity of CHO and E-coli derived rhBMP bioimplants in vitro and in vivo, and the efficacy of the different carriers in vivo.

1. To evaluate the source effect on rhBMP activity, the following experiments were performed:

   ➔ In vitro experiments comparing the potency of the two rhBMPs

   ➔ In vivo experiments comparing the activity of the two bioimplants

2. To evaluate the carrier effect on rhBMP activity, the following experiments were performed:

   ➔ In vivo experiments comparing the effect of the ACS and CaP carriers on the rhBMP activity.

2.2 Materials

The experimental groups consisted of mammalian derived rhBMP-2, and E-coli derived rhBMP-2. The E-coli derived rhBMP (CowellMedi bioimplant) utilized calcium phosphate granules as a carrier. Both mammalian derived rhBMPs (Induce and Medtronic Infuse bioimplant) were derived from Chinese hamster ovary cells and utilized the absorbable collagen sponge as a carrier (table 4).

RhBMP Source	*CHO*	*CHO*	*Bacterial*
	Induce	*Medtronics Infuse*	*CowellMedi*
RhBMP Carrier	ACS	ACS	CaP

Table 4: Bioimplants used in this study.

2.2.1 Measuring the amount of E-coli derived rhBMP

Unlike the mammalian derived bioimplants where the rhBMP was supplied separate from the carrier, the E-coli derived rhBMP (CowellMedi-BMP) was supplied in a bottle containing large granules (β-TCP 70%/HA 30% granules) and fine powder (lyophilized rhBMP-2) (figure 8). Based on the information provided by the manufacturer, the concentration of rhBMP was 1.25mg/mL upon reconstitution with PBS.

To determine if any rhBMP-2 was associated with the TCP/HA granules, the granules were separated from the powder and the amount of rhBMP was measured. The TCP/HA granules were poured out of the bottle according to the manufacturer's instructions, and transferred to a sterile 1.5mL eppendorf tube. Following this, 200uL of sterile PBS (phosphate buffered solution) was added to the remaining powder in the bottle, and this solution was then transferred to a separate sterile eppendorf tube for analysis.

0.2mL of PBS was added to the TCP/HA granules and was collected after 24 hours, at which point another 0.2mL PBS was added to the granules. After a second 24 hours, this solution was collected. Following this, the amount of rhBMP-2 present in the buffer incubated with powder, and with the granules was measured by an ELISA. Results are summarized in Table 5.

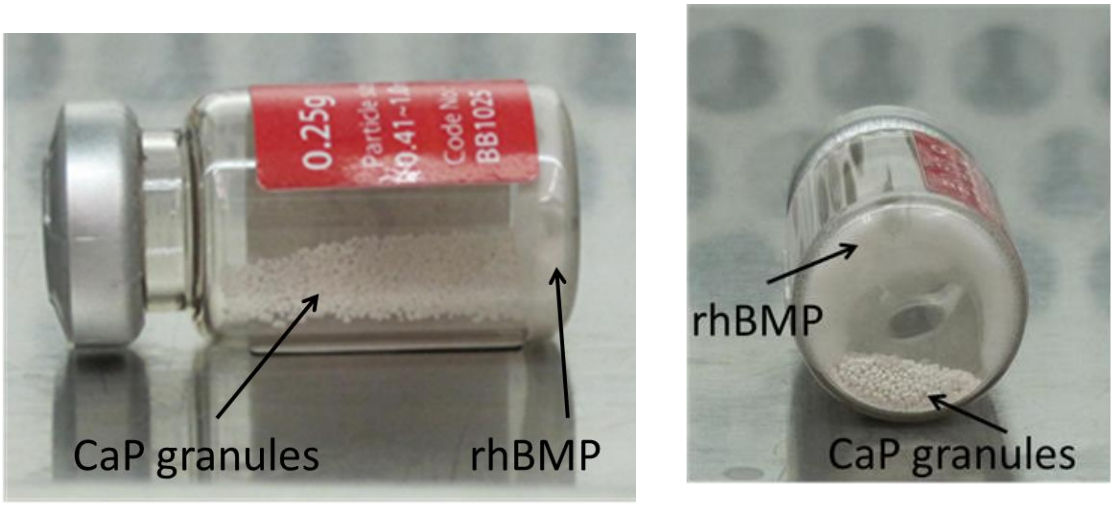


Figure 8: Sample of E-coli derived rhBMP (Cowell Medi) bottle with calcium phosphate granules (CaP), and rhBMP powder (rhBMP)

Sample	*Predicted Concentration*	*Dilution*	*Measured (ng/mL)*	*Estimated concentration*
Cowell powder	1.25mg/mL	10X10(e)6	0.334	3.34mg/mL
Cowell BCP 1st 24hr	0	100	0.518	52ng/mL
Cowell BCP 2nd 24hr	0	100	0.162	16ng/mL

Table 5: ELISA estimation of rhBMP-2 concentrations in the Cowell Medi kit

The concentration of rhBMP from the powder was significantly higher than the predicted concentration. This is most likely due to the ELISA over-estimating the E-coli rhBMP. While the absolute amount may not be accurate, the proportion of rhBMP in the powder compared to the rhBMP released from the CaP granules is expected to be. Consequently these results suggest that virtually all of the E-coli rhBMP is in the powder (>99.99%) with only very small amounts associated with the CaP granules. For the activity testing we assumed that 100% of the E-Coli rhBMP is present in the PBS solution applied to the powder at a concentration of 1.25mg/ml.

2.3Methods

2.3.1 In Vitro Experiments

Osteoinductive rhBMPs stimulate mouse muscle derived C2C12 cells to change their differentiation pathway from a myogenic to an osteogenic lineage [212, 213]. When C2C12 cells are cultured, they express very low levels of alkaline phosphatase activity.

Once exposed to rhBMP, C2C12 cells undergo osteoblastic differentiation, develop a cuboidal morphology and express high levels of alkaline phosphatase (ALP) activity. As the amount of BMP increases in the medium, so does the ALP activity of cells. The level of ALP is therefore used as an index of the osteogenic potential of the test agents.

The in vitro experiment compared the activity of two CHO rhBMPs (Induce BMP and Medtronic BMP) to that of E-Coli rhBMP (Cowell Medi) at different concentrations (25, 50, 100, 200, 400 ng/mL) with 3 samples per concentrations. ALP level, protein level and ALP/protein were measured at 30 minutes and 24 hours.

2.3.1.1 Cell culture of C2C12 cell line

2.3.1.1.1 Stock & Cultures

Frozen aliquots of C2C12 cells were thawed in a 37°C water bath from storage in liquid nitrogen. 14mL of prewarmed culture medium (α-minimal essential medium (αMEM) + 15% heat-inactivated fetal bovine serum (FBS)) was then added to the thawed 1 ml of cell suspension containing 10^6 cells/mL. Once the cells were suspending in the medium, they were plated into a T-75 flask and incubated at 37°C with 5% carbon dioxide. After monitoring and reaching 80% confluence (approximately 72 hours), the culture was subcultured to ensure that myogenic differentiation had not occurred, and that the cells had remained in an undifferentiated state.

Following this, the culture medium was removed and the cells were rinsed with warmed sterile phosphate buffered saline (PBS), and 5ml of 0.05% trypsin-EDTA solution was added in order to subculture the C2C12 cells. Phase contrast microscopy was used to monitor the progress of cell detachment. After complete cell detachment, 5mL of culture medium was added in order to neutralize the trypsin. The trypsinized cell suspension was then transferred to a test tube, and a 1 mL aliquot was placed into a ViCell cell counter (Beckman) to determine cell number. The ViCell cell counter verifies the viable proportion of cells, the aim being to have at least 95% of viable cells in the sample.

As the 1mL aliquot sample was being verified in the ViCell counter, the remainder of the mixture was centrifuged at 200g for 5 minutes. Once the supernatant was discarded, the cells were resuspended in a culture medium at a concentration of $0.5x10^5$ cells/mL. If the cells were to be used as a stock culture, 10mls of the cell suspension were seeded into a new T75 flask. However, if the cells were to be used for an assay, they were seeded into wells of a 24-well plate, at 1ml per well.

2.3.1.2 Alkaline Phosphatase and Protein Assays of Cell lysates

2.3.1.2.1 Alkaline Phosphatase (ALP) Assay

The ALP assay is based on a chemical reaction involving the conversion of p-nitrophenol phosphate (pNPP) to p-nitrophenol (pNP) and inorganic phosphate while in the presence of ALP. At a temperature of 37°C and pH of 10.5, ALP hydrolyses pNPP to pNP and free phosphate. Before hydrolysis, the pNPP solution is colourless. The effect of hydrolysis makes the end product (pNP) appear yellow (figure 9).

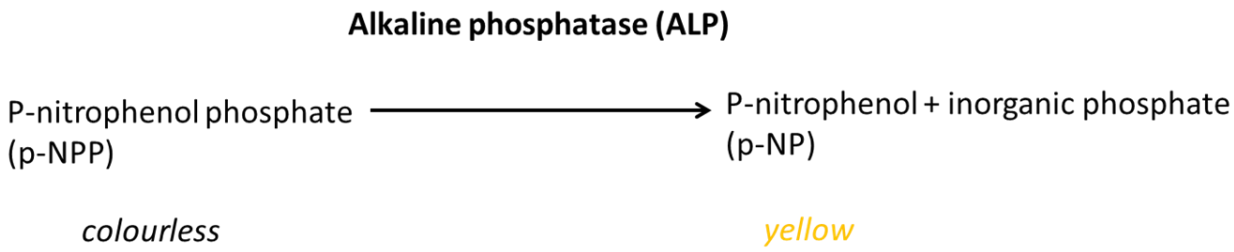


Figure 9: The conversion of p-nitrophenol phosphate to p-nitrophenol and inorganic phosphate in the presence of ALP.

As the ALP concentration increases, a greater production of pNP results in a more densely coloured solution. Compared to pNP standards, the more densely coloured solution will display greater absorbance at 405nm.

Prior to running the assay, preparation of reagents and pNP standards was required. The first step in preparing the reagent was to warm the phosphatase substrate (Sigma 104 – 100mg capsules pNPP) to room temperature. The contents of the capsules were then separated and emptied into a dark tinted bottle. After diluting the solution with

25mL of laboratory grade water per capsule, the dissolved pNPP substrate was mixed with 221 alkaline buffer solution (2-amino-2-methyl-1-propanol, 1.5mol/L, pH 10.3) in equal portions (1:1) and subsequently stored in a darkened bottle at 4°C.

The pNP standards were prepared as follows: a stock solution of 200µL p-nitrophenol (pNP, 10µmol/mL) was diluted in 10mL of 0.02M sodium hydroxide (NaOH). Serial 1:1 dilutions were performed to generate a series of solution standards: 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 nmol/mL. With two wells per standard, two hundred and forty microliters (240µl) of each standard were pipetted into a standard 96 well assay plate.

Thawed cell lysates were then vortexed and held on ice. Twenty microliters (20µl) aliquots were then pipetted into the 96 well plate wells with 4 wells per sample. An additional blank sample of cell lysis buffer alone was included to correct for the low level absorbance caused by the reagent buffer. Two hundred microliters (200uL) of the pNPP substrate buffer was then added to each of the samples and blank wells (but not the standards). At a temperature of 37°C, the 96 well plate was incubated for 30 minutes to 24 hours.

Using a plate reader (Versamax, Molecular Devices), the absorbance was then read at 405nm.The ALP was measured after 30 minutes and 24hours of incubation time. By comparing with the absorbance from the pNP standards, the amount of alkaline phosphatase activity was calculated. The mean of the 4 aliquots measured for each sample was taken as the result for each sample. The results were reported in units of activity (U) with each until being equivalent to 100nmol pNP produced per 20µl sample per 30 minutes.

2.3.1.2.2 Protein Assay (Coomassie)

A protein assay was performed in order to normalize the results of the alkaline phosphatase activity to the number of cells in each well.

By diluting the 2mg/ml bovine serum albumin (BSA) from stock solutions provided with the protein assay kit (Coomassie Plus Kit, Fisher Scientific, Mississauga ON) with cell lysis buffer, protein standards were prepared. With 4 wells per sample/standard, twenty microliters aliquots of each sample or standard were pipetted into the 96 well plate. Coomassie Plus reagent reacts with the protein in the samples turning them from a brown to a blue colour. Two hundred microliters (200µl) of this reagent was added to each well. After 5 minutes, the absorbance at 595nm was determined by the microplate reader. By comparing the absorbance of the samples with that of the known concentrations of the standards, the amount of protein in each sample was determined. The value obtained from the ALP assay was divided by the mean protein concentration obtained for the same sample, in order to normalize the ALP activity of the samples. The normalized results were reported as U/µg protein.

2.3.2 In Vivo Experiments

2.3.2.1 Experimental Groups

Table 6 summarizes the experimental groups in vivo.

	Carrier	Source	N (number of samples)	Dose (ug)
Group 1	ACS	---	6	0
Group 2	ACS	Induce	6	5
Group 3	ACS	Induce	6	20
Group 4	ACS	Medtronic	6	5
Group 5	ACS	Medtronic	6	20
Group 6	ACS	CowellMedi	6	20
Group 7	CaP	---	6	0
Group 8	CaP	Induce	6	5
Group 9	CaP	Induce	6	20
Group 10	CaP	CowellMedi	6	20

Table 6: Experimental groups in vivo. ACS = absorbable collagen sponge, CaP= calcium phosphate granule.

2.3.2.2 Experimental Design

The in vivo design used in this study was based on previous studies involving insertion of a bioimplant containing 50µg of rhBMP into a mouse muscle pouch [48, 214, 215]. For this investigation, the protocol utilized male CD-1 mice aged 37 to 45 days old, weighing between 27 and 32 grams, divided into 10 groups, with 6 samples per group (n=6). In 6 of the groups, the ACS carrier was used, while in the other 4 groups the CaP carrier was used. The animals received one bioimplant in the right thigh and one bioimplant in the left thigh.

2.3.2.3 Fabrication of the Bioimplants

2.3.2.3.1 Sterilization of the carrier

The control side in the ACS groups consisted of an empty #5 gelatin capsule containing collagen sponge alone, which had been sterilized over chloroform vapour for 4 hours using bell jar/desiccator. The bell jar was then opened, and the pouches were retrieved and placed in a BSC (biologic safety cabinet) overnight to air out the chloroform vapours. The CaP capsules were sealed and placed into a sterilization pouch to be sterilized above chloroform vapours for 4 hours in a bell jar/desiccator, retrieved and left to air out overnight in a biologic safety cabinet.

2.3.2.3.2 Medtronics rhBMP-2 Bioimplant and Induce rhBMP-2 Bioimplant

Medtronic's Infuse® is designed to be used as a reconstituted powder added to an absorbable collagen sponge (ACS). Infuse bone graft was supplied in a kit with all the necessary components: a vial with the lyophilized rhBMP-2, a vial with sterile water, ACS, syringes and needles. The rhBMP-2 was provided as a lyophilized powder in vials of 1mg, 4.2mg, or 12mg of protein. According to the manufacturer, each milliliter of rhBMP-2 solution contained 1.5mg of rhBMP-2, 5mg of sucrose NF, 25mg of glycine USP, 3.7mg of L-glutamic acid FCC, 0.1mg of NaCl USP, 0.1mg of polysorbate 80 NF, and 0.1mL of sterile water. The solution was diluted in water to 1mg/mL. This solution had a pH of 4.5, and was clear and colorless. The package was stored at room temperature (figure 10).

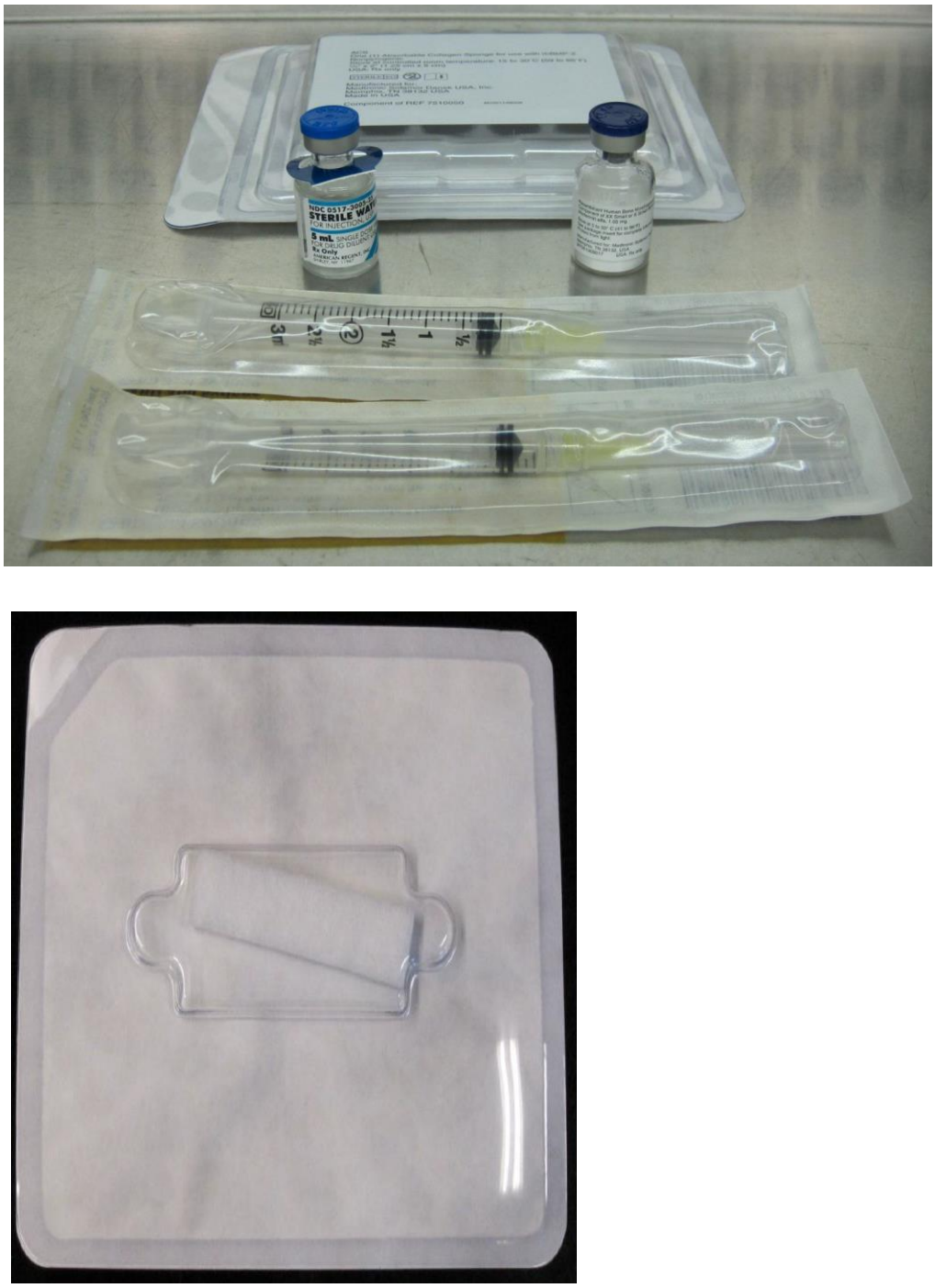


Figure 10: Infuse kit: A vial with the lyophilized rhBMP-2, a vial with sterile water, the syringes (above), and the ACS (below)

The Infuse rhBMP-2 was prepared at the time of surgery by reconstituting the lyophilized rhBMP-2 with sterile water and applying it to the ACS. According to the manufacturer's instructions, the first step in preparation of the bioimplant was to open the outer package of the ACS on the non-sterile field, and place the inner sterile package containing the 1" X2" collagen sponges on the sterile field. One of the two 10mL syringes/needles was also placed on the sterile field. 8.4mL of sterile water was to be withdrawn with the second syringe, and reconstituted with the rhBMP-2. In the sterile field, the 10mL syringe was used to withdraw 4mL of reconstituted rhBMP-2 from the vial and uniformly distribute it on three 1"X2" collagen sponges. The wetted collagen sponges were allowed to stand for a minimum of fifteen minutes before implantation. The bone graft was not to be used after 2 hours of reconstitution.

Induce rhBMP-2 bioimplant was available from the frozen stock aliquot with a final concentration of 1mg/mL.

The ACS (absorbable collagen sponge) was soft, white and pliable. The ACS (1"X2") was cut into 40 pieces of 5mmX 5mm in order to fit into the #5 gelatin capsules. At the time of surgery, the gelatin capsule cap was removed, and the half capsule containing all the collagen sponge was placed into the implant site positioned pointing down. The amount of BMP added was carefully pipetted into the capsule and the wound was closed.

2.3.2.3.3 CowellMedi Bioimplant

The Cowell-BMP bioimplant was supplied in a bottle. This bottle contained large granules and fine powder. According to the manufacturer's instructions, the CaP granules were poured out of the bottle into a sterile 15mL eppendorf tube as the rhBMP-2 stays stuck to the bottom of the bottle. The concentration of rhBMP-2 was reported to be 1.25mg/mL. Sterile PBS was carefully added to the bottle and pipetted up and down (without creating bubbles) to ensure uniform solution of 1mg rhBMP/mL. The Cowell-BMP was then aliquotted into 2 sterile eppendorf tubes and stored at -20^0C. The 10mg CaP granules were weighed, and placed into each gelatin capsule. Half gelatin capsule containing the CaP granules was placed into the implant site positioned pointing down, and the correct amount of rhBMP was pipetted into the capsule.

2.3.2.4 Surgical phase

2.3.2.4.1 Implant placement

The surgeries were performed in the animal surgical suite at the Faculty of Dentistry at the University of Toronto, Ontario. The mice were labeled with ear tags, and anesthetized using 4% isoflurane inhalational anesthetic in 1L of oxygen via a nasal cone. Anesthetic maintenance was achieved with 2% isoflurane with 0.8L of nitrous oxide and 0.6L of oxygen. Each mouse received 0.03mL of the analgesic, buprenorphine, subcutaneously before the surgery. Electric clippers were used to remove fur over the dorsal aspect of the pelvis and hind quarters. The skin was prepared using proviodine solution, and the animal draped using disposable sterile paper drapes.

A #10 scalpel blade was used to make a 1.5cm incision along the dorsal midline through skin and subcutaneous tissue. Blunt dissection was performed to gain access to the site of implantation. Dissection beneath the gluteus superficialis muscle then followed, and the muscle pouch was developed. The capsule was inserted pointing down (figure 11). The unsealed capsules were inserted dry and the aqueous rhBMP-2 was then added to either the ACS or CaP within the open capsule via a micropipette (figures 12-13). The muscle pouch was gently repositioned, ensuring coverage of the capsule, and the skin wound was reapproximated and closed using 2-3 surgical clips (figure 14). Each subject was then allowed to recover in its original cage population. The cages were then returned to the small mammal dormitory of the animal care facility.

The morning after the surgery, the mice were once again given 0.03mL of the analgesic, buprenorphine subcutaneously, and were checked to ensure no wound dehiscence or complications had occurred. Their progress was monitored regularly throughout the next 28 day period as all subjects appeared well. On post-op day 4, wound clips were removed. All subjects regained excellent mobility of the hind legs, and no subjects demonstrated any post-op complications.

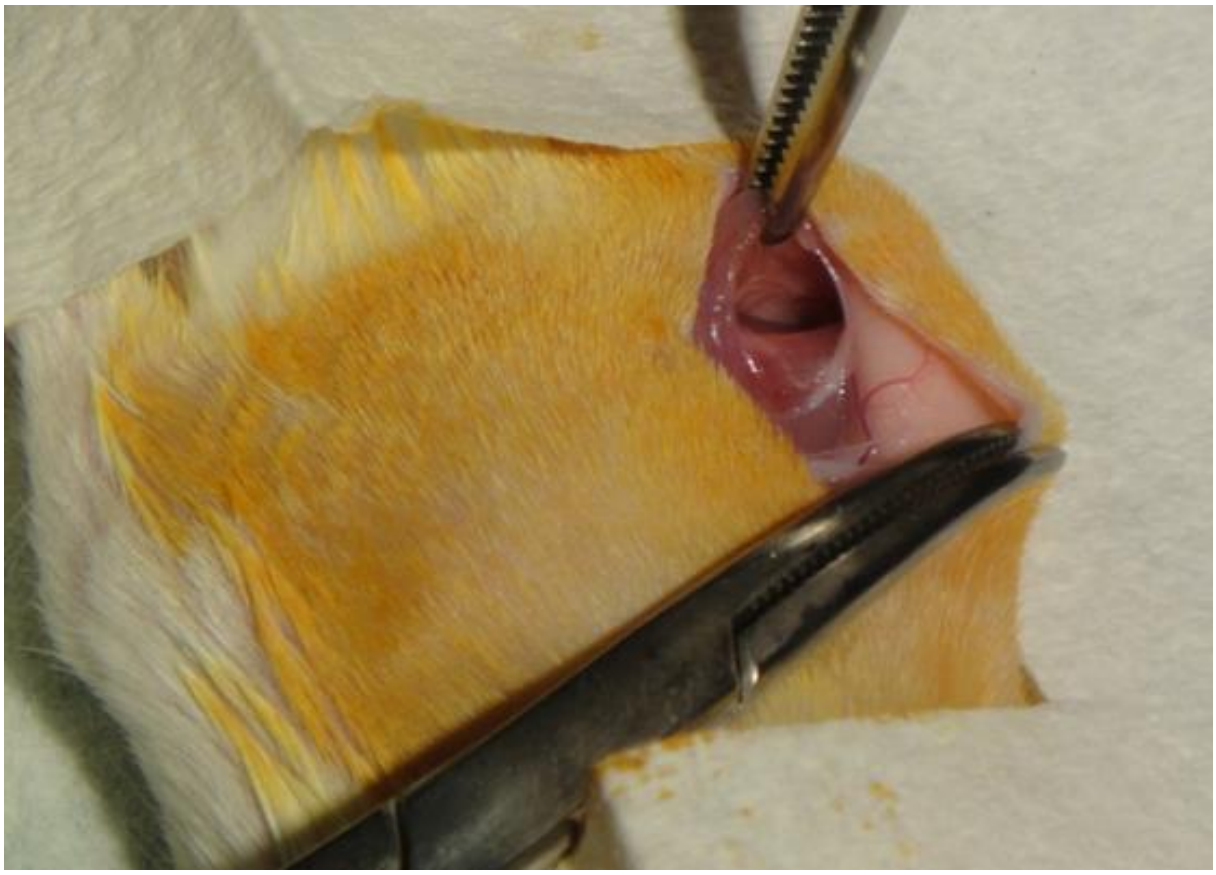


Figure 11: Hind quarter muscle pouch with inserted gelatin capsule

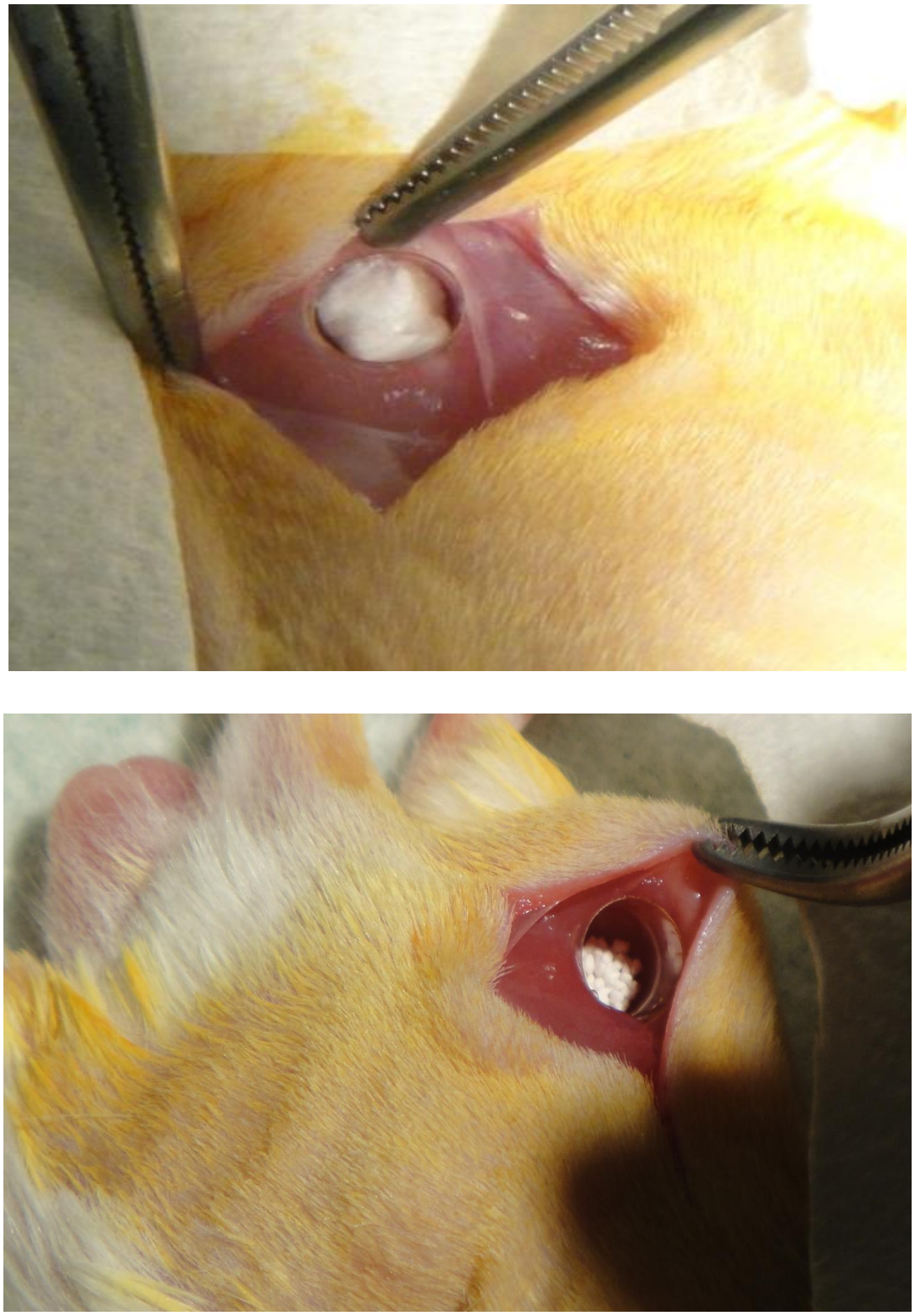


Figure 12: Absorbable collagen sponge (top) and calcium phosphate granules (bottom) within the gelatin capsule

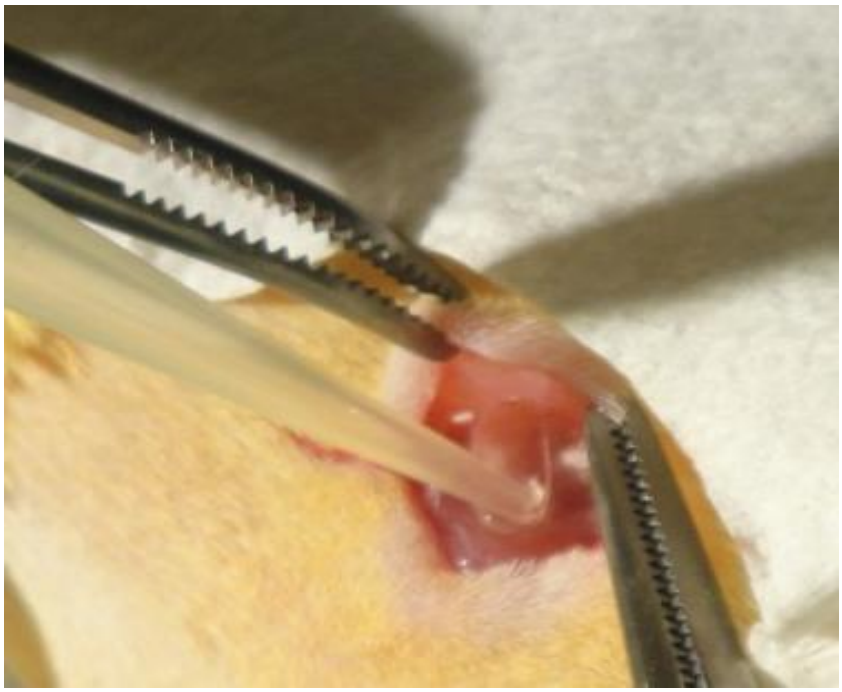


Figure13: BMP-2 added via micropipette to the gelatin capsule

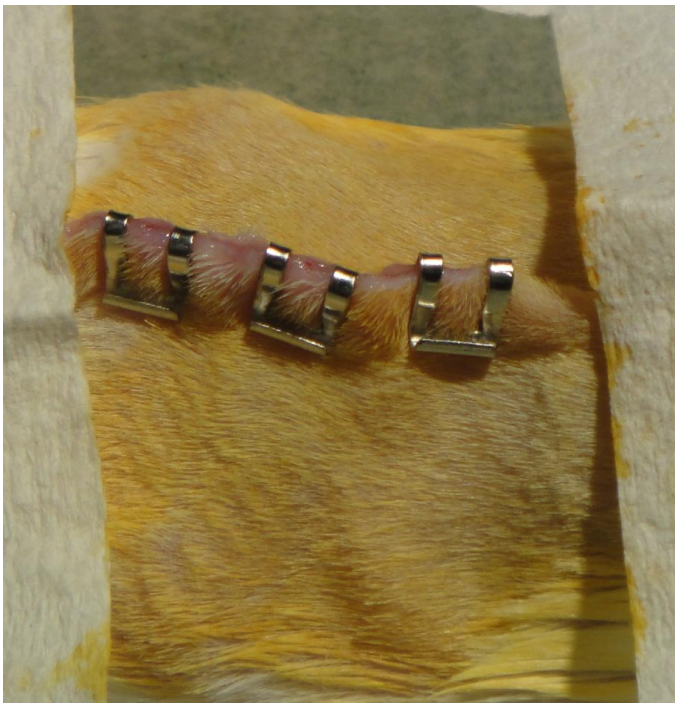


Figure 14: Closure of wound with surgical clips

2.3.2.5 Sample Harvest

On post-op day 28, the subjects were euthanized using a sealed carbon dioxide chamber, then underwent cervical dislocation. With the aid of surgical scissors, the skin from the hind quarters was dissected off. A bone cutting forceps was then used to sever the spinal column cephalad to the pelvis, and remove the feet at the level of the ankles. Each sample was placed into a labelled 50 mL sealable polypropylene test tube (BD Falcon, Canada) containing 10% buffered formalin tissue fixative.

2.3.3 Radiographic Evaluation

2.3.3.1 CT protocol, calibration and reconstruction

The General Electric Healthcare Explore Locus SP microCT scanner was used to image the specimens. The scanner consists of two specimen tubes for scanning both large and small diameter tubes. The larger diameter tubes were required due to the size of the mouse hindquarter specimens. Oriented with the pelvis at the inferior aspect, the specimens were individually placed into the large standardized tubes. The tube was packed with moistened cheesecloth in order to stabilize the specimen and ensure its minimal shifting during the scan.

Initially the scout scan gave an overall image from which an area of interest was selected for scanning at full resolution (30 um). The manufacturer's protocol "short scan, large tube, 70 minutes", was used for the full resolution scan. A GE calibration block was included with each scan for image calibration at reconstruction. This block contained areas reflective of the consistency of air, water and cortical bone. The bone standard, referred to as SP3 or Gammex, is designed such that calibration value approximates 3500 Hounsefield units. This translates into a bone mineral density of 1050mg/mL of hydroxyapatite. Once the scans were completed, the GE Healthcare Explore Microview v.2.0 software was used to select the area of interest to create a final reconstruction at half resolution (60 um) in order to reduce reconstruction time and file size. This gave a resulting image of 60 um resolution.

Inter-scan variance was assessed using each sample through the calibration block in order to act as an internal control. The analysis of variance in the value of the calibration block was undertaken in order to ensure results that were not due to variance in scan. Standard deviation and coefficient of variance were determined based on the calculation of the mean values of air, water and bone.

2.3.3.2 Image formatting and selection of Region of Interest

A region of interest (ROI) was determined, and outlined by a technique known as splining. The ROI was meant to encompass all areas containing newly formed bone (the ossicle). Splining involves outlining, in a peripheral halo technique, the bony mass with the region of focus on each individual CT slice. Contouring the edge of the bony mass, a series of points are manually selected. The software then connects these points, revealing the external contour of the bony mass (figure 15).

Each specimen provided a CT image comprised of approximately 700 slices. The manual contouring, or splining, was performed approximately every 3-5 slices with the auto-splining interpolating the contours of the remainder. The splining process was completed by one individual. The same individual also reviewed the auto-splining slice to verify the accurate computer generated contour inclusion of new bone, and not native bone. The resulting ROI is a 3-dimensional image of the newly formed bony mass (figure 16).

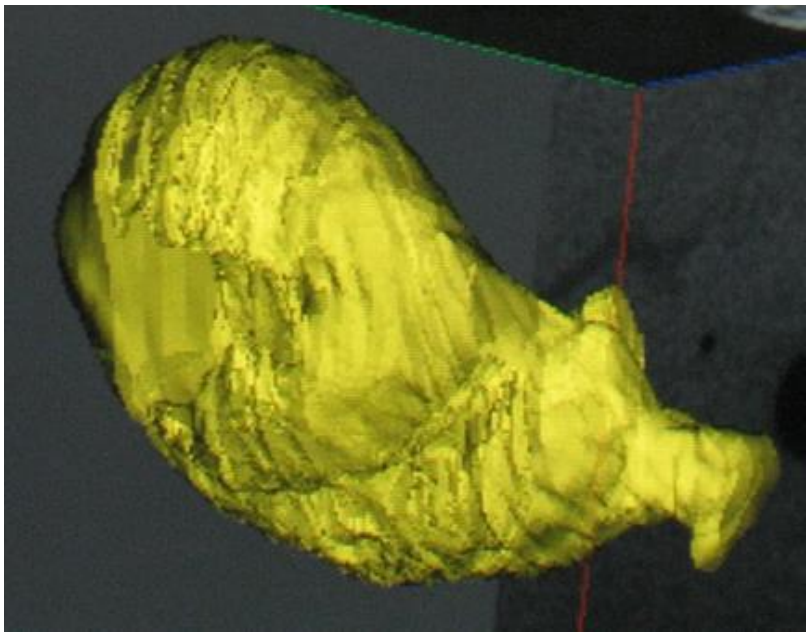


Figure 15: External contour of bony mass

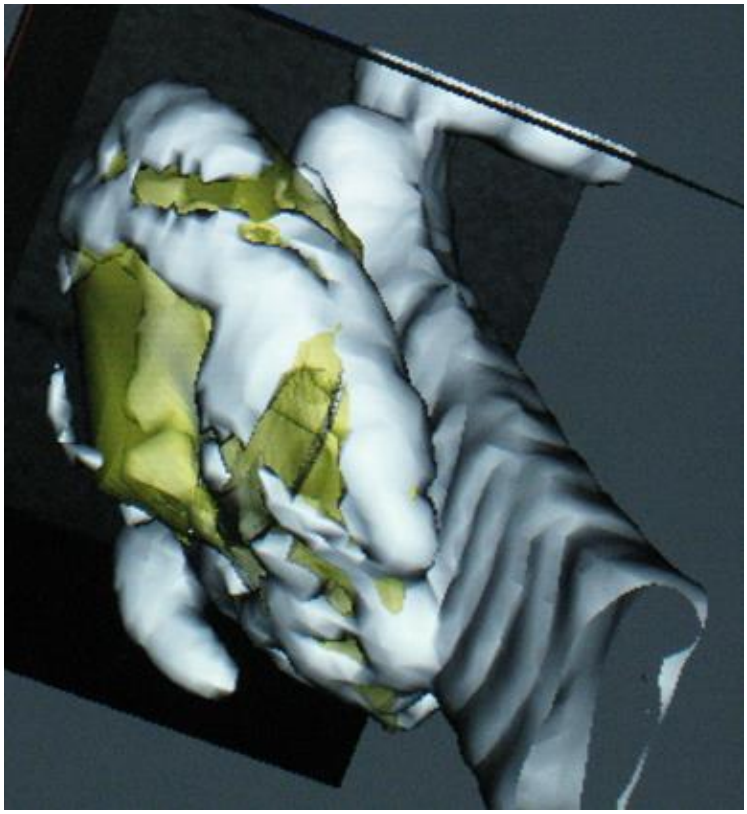


Figure 16: 3D reconstruction of bone mass

2.3.3.3 Threshold Value

The computed tomography (CT) images obtained were based on the interaction of x-ray photons and the tissues they encountered, and the degree to which the tissues blocked the photons. Each tissue type is assigned a different attenuation coefficient, which is a reflection of its density, such that the greater density tissue (eg: bone) will have a higher attenuation coefficient than the lower density tissue (eg: adipose tissue).

The first images to be obtained were single two dimensional images comprised of pixel values. Each of these pixel values were assigned an arbitrary density unit (ADU). Once the 3D volume reconstruction was performed from the 2D images, the pixels were represented by a volume rather than a point. These volumes are referred to as voxels, and the newly converted ADUs are converted to CT units. Following this, these CT units were calibrated to Hounsfield units (HU), which is the standardized unit of CT contrast. The calibration was performed by comparing to known standards of air, water and bone, and the use of a calibration block [216] (Table 7)

	Air calibration value	Water calibration value	Bone calibration value(HU)
Mean	2.100	9.559	3392.095
Standard deviation	0.135	0.298	210.095
CV	6.426	3.112	6.194

Table 7: Standard values for air, water and bone calibration.

To analyze the quantity and quality of bone within in the ROI, CT images were segmented into non-bone and bone phases. After reconstruction of the scanned segments, the ROI included both bone and other tissues. Individual voxel greyscale values were compared against a set of threshold values in order to complete segmentation [217].

The bone/non-bone boundary is not a discrete interface but a gradient of greyscale values. The voxels that were included in the bone phase had values equal to or greater than the threshold value, whereas the voxels included in the non-bone phase had values less than the threshold value. In order to set our threshold value, we first evaluated the calibration values of the standardized calibration block. By utilizing several trial threshold values to analyze the samples, we decided on a value that maximised the bony volume without including other tissues. The upper limit value used was 3500 HU, whereas our lower limit value was 300 HU.

2.3.3.4 Image Analysis

Bone analysis was then performed on the ROI. The bone analysis functions included total volume (TV), bone volume (BV), bone mineral density (BMD), bone mineral content (BMC), tissue mineral density (TMD), tissue mineral content (TMC), and bone volume fraction (BVF). A description of these parameters is outlined in table 8.

Unlike DEXA (dual-energy xray-absorptiometry) where the mineral density is considered a value of the quantity of bone, in the three dimensional microCT analysis, the values for mineral density (BMD, TMD), represent the mineralization of the bone tissue that is being analyzed, and are considered values of the quality of the bone.

In 2011, Humber et al published a microCT method that allowed for correction of the presence of CaP scaffolds when estimating the amount of bone present by microCT. This is possible as the CaP scaffolds have significantly higher radio-density than bone. By measuring the various microCT parameters at 2 different threshold values, a lower threshold that includes the bone and CaP, and an upper threshold that includes only the CaP, the contributions due to the CaP can be subtracted from the combined measurements to determine the amount of bone present in the samples [218].

Parameter	Definition	Threshold Dependant	Quantity vs. Quality
Total Volume (TV in mm^3)	Includes the total volume selected for analysis. Includes all volume assumed by bone, soft tissues, scaffold and fluids	No	Quantity
Total Bone Volume (BV in mm^3)	Determines the volume of bone in the surgical sites. Includes voxels greater than the threshold value	Yes	Quantity
Bone Mineral Content (BMC in mg)	Determines mineral (calcium) content within the region of interest	No	Quantity
Bone Mineral Density (BMD in mg/mm^3)	Determines the mineral density within the region of interest (BMC/TV)	No	Quality
Tissue Mineral Content (TMC in mg)	Determines mineral (calcium) content of tissue within the ROI with voxels greater than the threshold value	Yes	Quantity
Tissue Mineral Density (TMD in mg/mm^3)	Determines the mineral density within the tissue with voxels greater than the threshold value (TMC/BV)	Yes	Quality
Bone Volume Fraction	Compares the fraction of bone greater than the threshold value to the total volume within the ROI (BV/TV)	Yes	Quality

Table 8: Bone microCT parameters

$aBV = BV_{LT} - BV_{UT}$
$aTMC = TMC_{LT} - TMC_{UT}$
$aTMD = aTMC/aBV$
$aBVF = aBV/TV$

Table 9: Explanation of adjusted values: aBV- adjusted bone volume, LT- lower threshold, UT- upper threshold, aTMC- adjusted tissue mineral content, aTMD- adjusted tissue mineral density, aBVF- adjusted bone volume fraction.

In the current study, the bone parameters that were considered to be threshold dependent (BV, TMC, TMD, BVF) were values that had been adjusted for the presence of the scaffold. These adjusted values were more accurate in interpreting the quantity and quality of new bone formed. (Table 9)

In order to analyze the quantity of bone formed, the total volume, and the adjusted bone volume values were used. The adjusted bone volume represented the quantity of bone in the region of interest with voxels greater than the threshold value. The adjusted tissue mineral density was used to reflect the quality of the bone formed. The adjusted tissue mineral density represented the adjusted values for the density of bone.

2.4 Statistics

2.4.1 Sample populations and Comparison of means

All of the CT data was analyzed using the SPSS 20 IBM statistical software. The data was evaluated for normality and equal variance, and statistical analysis was performed using the one-way ANOVA and post-hoc testing. With significance established at $p<0.05$ for each CT analysis category, the tests were carried out to assess for statistical significant differences between the E-coli derived rhBMP-2 bioimplant and the CHO derived rhBMP-2 bioimplant.

2.5 Histological Evaluation

After microCT analysis, specimens were prepared for histological evaluation. The fixed specimens were first decalcified in a solution of 45% formic acid in 20% sodium citrate for 6 weeks. In preparation for serial sections through the bony spicule, the specimens were embedded in paraffin wax. Sections were cut perpendicular to the long axis of the native femur, and were stained with hematoxylin and eosin. The individual sections from each specimen were then examined under light microscopy in order to confirm the makeup of the induced tissue.

3 Results

All data in the results section is available in table format in the appendix section.

3.1 In vitro results

3.1.1 In Vitro comparison of E-coli rhBMP-2 to the two CHO rhBMP-2

Figure 17 summarizes the in vitro results.

E-coli rhBMP did not show significant activity, even at the highest doses tested (400ng/mL) at 30 minutes and at 24 hours.

At 25ng/mL, Induce rhBMP was statistically significantly more active than E-coli rhBMP ($p<0.05$).

Above 100ng/mL, both CHO rhBMPs (Medtronic Infuse and Induce rhBMP) stimulated significantly higher ALP activity at 30 minutes, and at 24 hours, compared to the E-coli rhBMP (CowellMedi) ($p<0.05$).

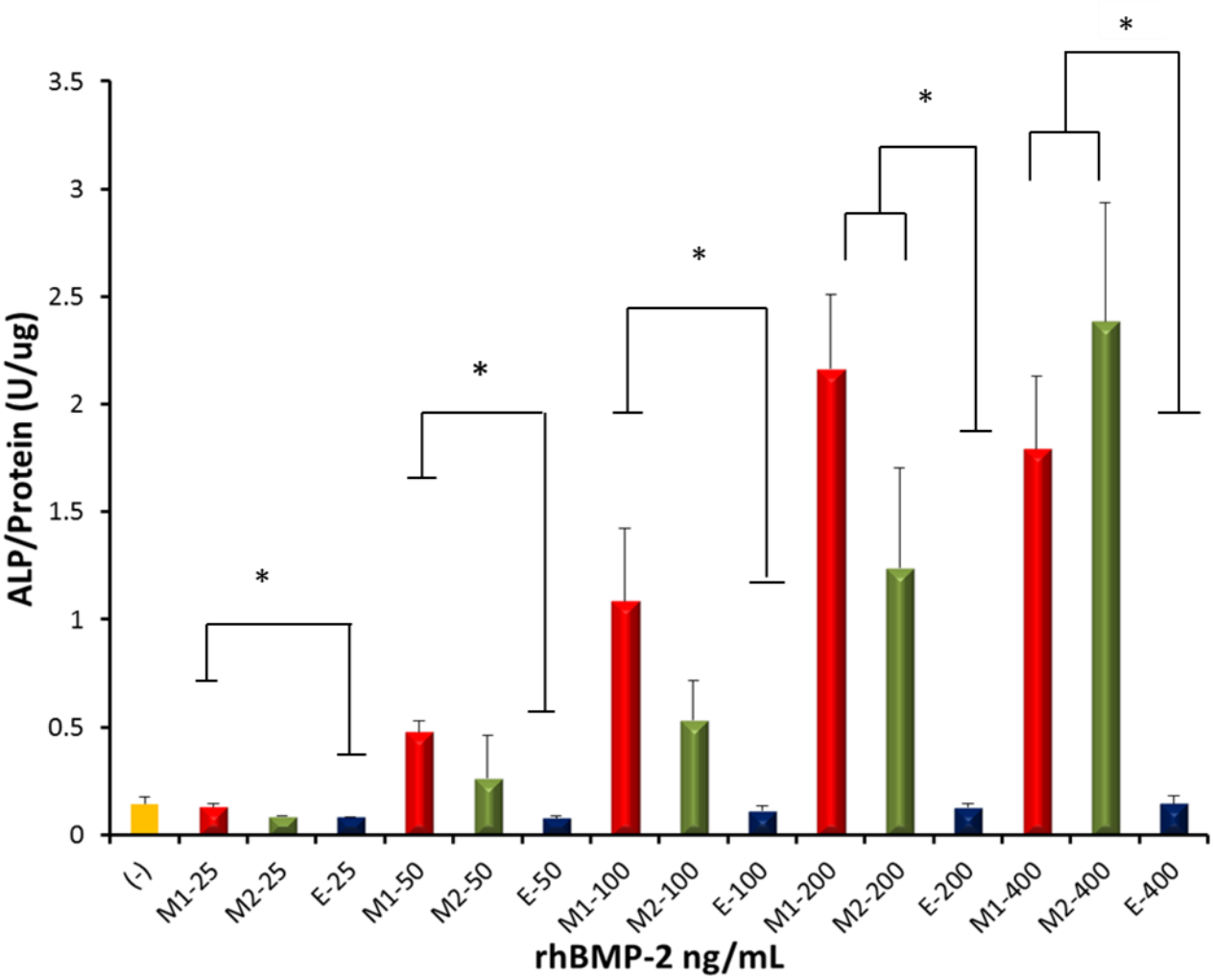


Figure 17: Bar graph demonstrating ALP/protein activity in the CHO and E-coli groups at 30 minutes.

M1-mammalian rhBMP (Induce); M2-mammalian rhBMP (Medtronic); E-E-coli rhBMP (CowellMedi);(-)- control.* represents statistical significant difference ($p<0.05$)

3.2 In Vivo results

3.2.1 Necropsy

During the harvesting of samples, there appeared to be significant ossicle formation with both the CHO rhBMP and the E-coli rhBMP, independent of the carrier type. Subjective assessment of the ectopic ossicle could not determine the difference in tissue volume or tissue quality between bioimplants.

3.2.2 Micro CT Analysis

To demonstrate the quantity of bone formed in vivo, the total volume and the adjusted bone volume values were used. The adjusted bone volume values were adjusted for the presence of the scaffold.

To describe the quality of the bone formed in vivo, the adjusted tissue mineral density values were used. These values were adjusted for the presence of the scaffold.

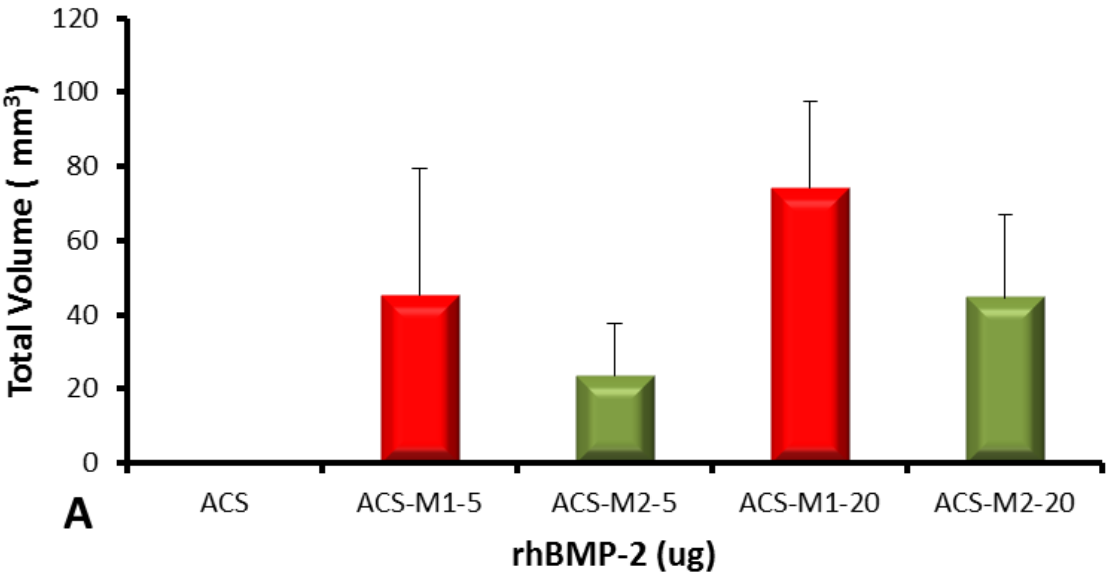
3.2.2.1 The Source effect on rhBMP activity

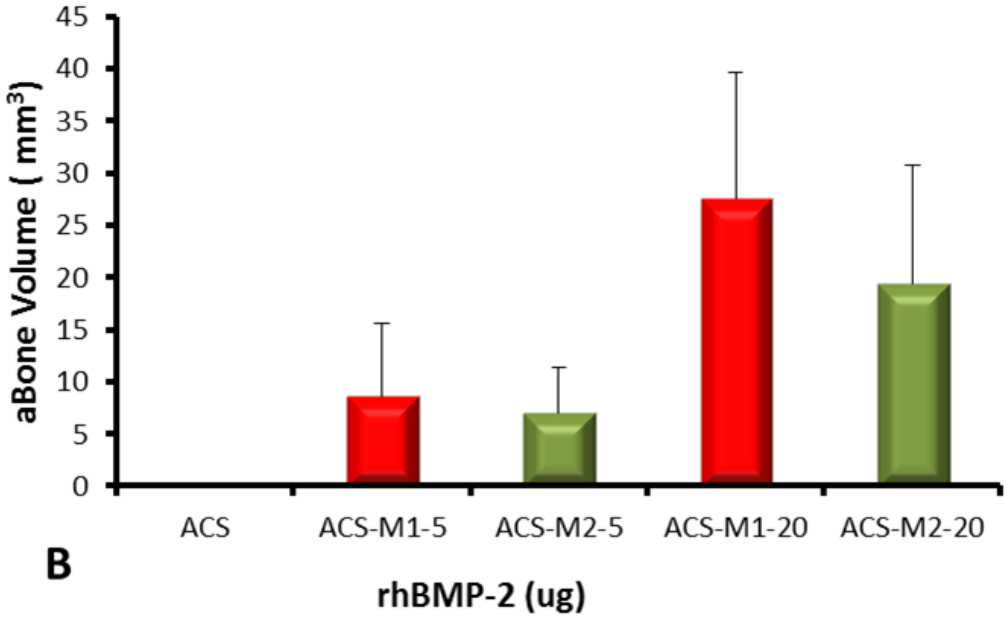
3.2.2.1.1 Comparison of the two CHO rhBMP bioimplants

The two CHO rhBMPs were compared using the ACS carrier. There was no significant difference in the total volume, or the adjusted bone volume, at 5 or 20ug of rhBMP comparing the Induce group (M1) and the Medtronic Infuse group (M2) ($p>0.05$) (figures 18A,B).

The difference in the adjusted tissue mineral density between the Induce group and the Medtronic Infuse group at both 5 or 20 ug of rhBMP was not statistically significant ($p>0.05$) (figure 18 C).

Based on this, we can conclude that there was no statistically significant difference in osteoinductivity between the two mammalian rhBMPs (Medtronic Infuse rhBMP and Induce rhBMP) at the doses tested (5 and 20ug)(figure 18)


120
100
80
60
40
20
0
Total Volume (mm3)
ACS
ACS-M1-5
ACS-M2-5
ACS-M1-20
ACS-M2-20
A
rhBMP-2 (ug)


45
40
35
30
25
20
15
10
5
0
aBone Volume (mm3)
ACS
ACS-M1-5
ACS-M2-5
ACS-M1-20
ACS-M2-20
B
rhBMP-2 (ug)

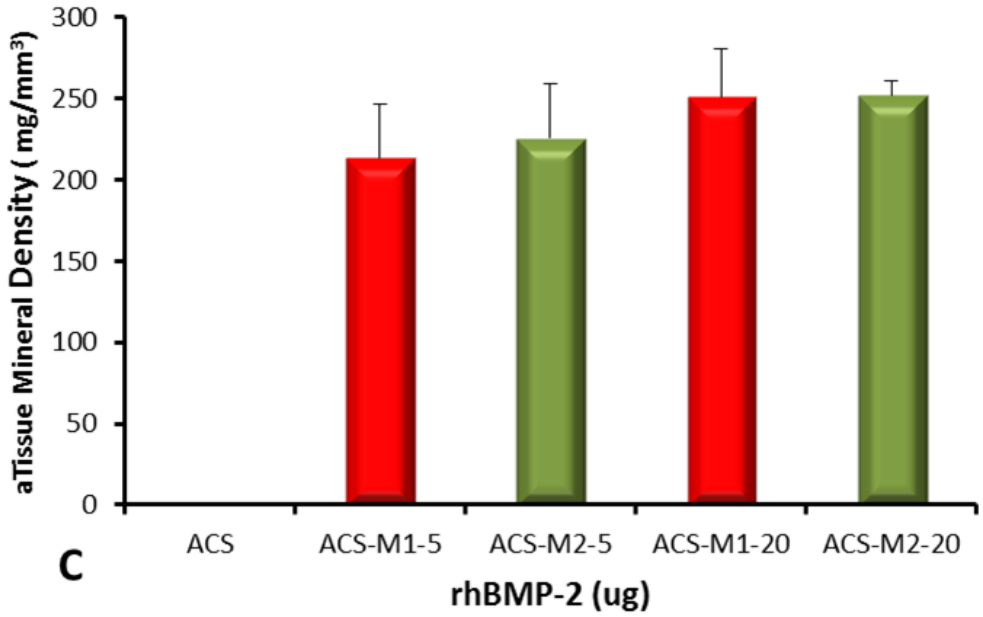


Figure 18: Difference in the total volume of bone induced (A), adjusted bone volume (B), and adjusted tissue mineral density (C) in Induce (M1) and Medtronic Infuse (M2) treated mice with 5ug and 20 ug of rhBMP at 28 days.($p>0.05$)

3.2.2.1.2 Comparison of CHO rhBMP to E-coli rhBMP

At 20ug of rhBMP, the total volume in the CHO rhBMP group was statistically significantly higher than in the E-coli rhBMP group, on both carriers ($p<0.05$) (figure 19A).

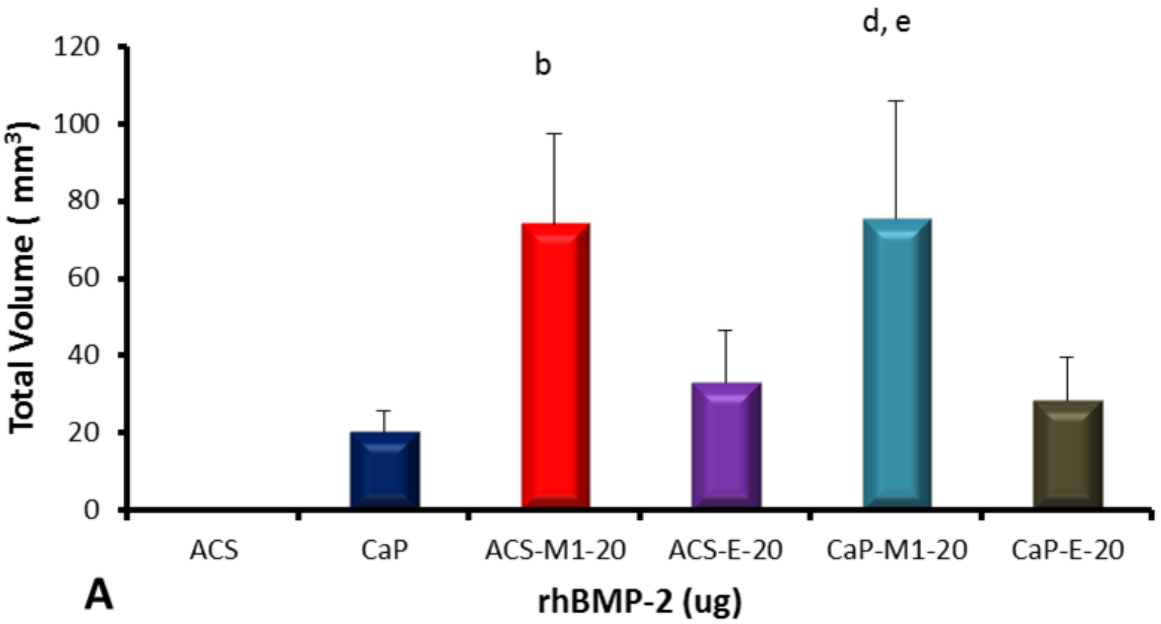
At 20ug of rhBMP, on the CaP carrier, the adjusted bone volume in the CHO rhBMP group was statistically significantly higher than in the E-coli rhBMP group ($p<0.05$). Compared to the CaP alone, the CHO rhBMP on the CaP carrier revealed statistically significant higher values in adjusted bone volume, and total volume ($p<0.05$) (figures 19A,B).

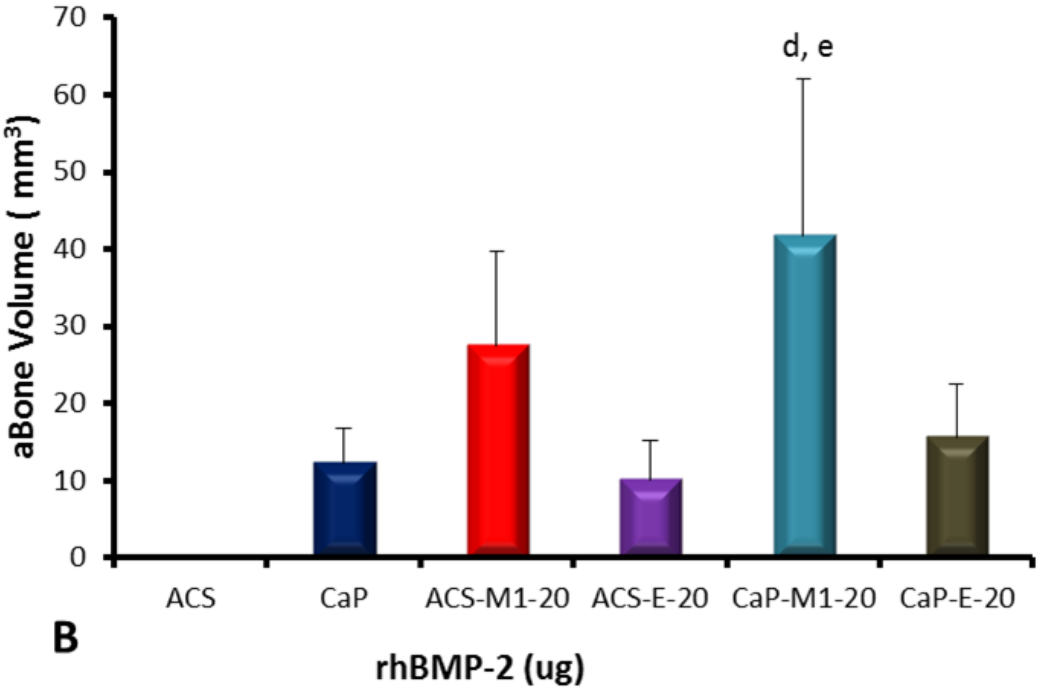
Hence, at 20ug of rhBMP, when paired with the CaP carrier, the CHO rhBMP produced a statistically significantly higher quantity of bone than E-coli rhBMP ($p<0.05$).

At 20ug of rhBMP, on the CaP carrier, the adjusted tissue mineral density in the E-coli rhBMP group was statistically significantly higher than in the CHO rhBMP group ($p<0.05$). CaP alone produced bone of statistically significantly higher adjusted tissue mineral density than CHO rhBMP on the CaP carrier ($p<0.05$) (figure 19C)

Hence, at 20ug of rhBMP, when paired with the CaP carrier, the E-coli rhBMP produced a statistically significant higher quality of bone than CHO rhBMP ($p<0.05$) (figure 19C)

Therefore, when paired with the CaP carrier, CHO rhBMP produces a higher quantity of bone than E-coli rhBMP, but E-coli rhBMP produces a higher quality of bone than CHO rhBMP ($p<0.05$).


d, e
b
120
100
80
60
40
20
0
Total Volume (mm3)
ACS
CaP
ACS-M1-20
ACS-E-20
CaP-M1-20
CaP-E-20
A
rhBMP-2 (ug)


70
60
50
40
30
20
10
0
d, e
aBone Volume (mm3)
ACS
CaP
ACS-M1-20
ACS-E-20
CaP-M1-20
CaP-E-20
B
rhBMP-2 (ug)

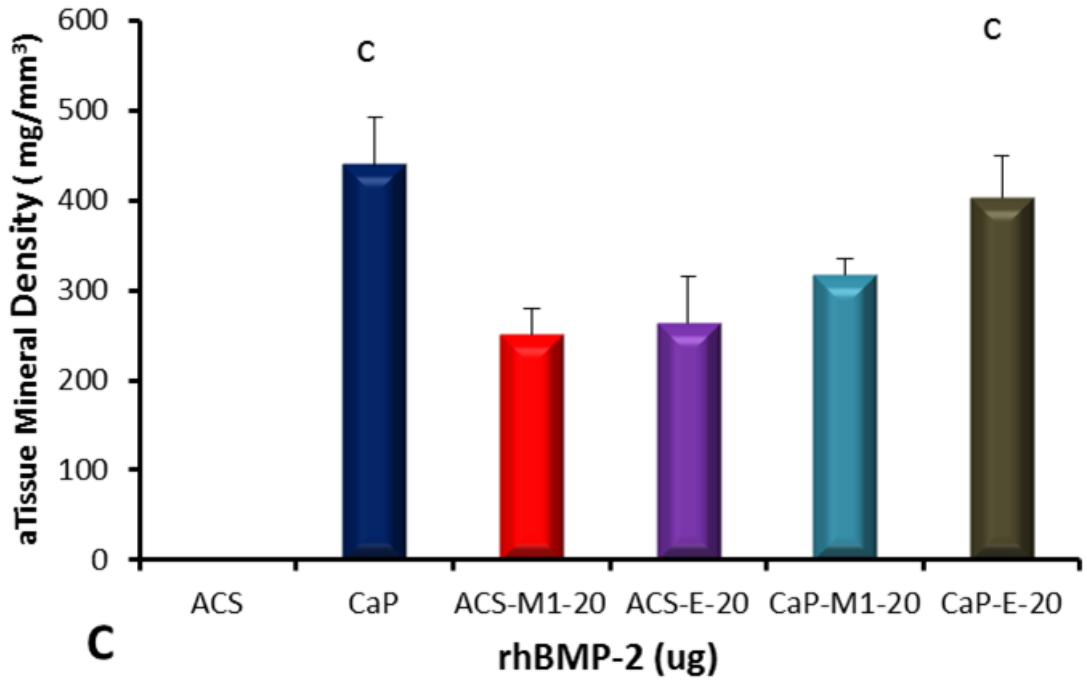


Figure 19: The difference in total volume (A), adjusted bone volume (B), and adjusted tissue mineral density (C) at 20 ug between the mammalian rhBMP and the E-coli rhBMP on the ACS and the CaP carriers at 28 days.

a: significantly different than ACS-M1-20

b: Significantly different than ACS-E-20

c: Significantly different than CaP-M1-20

d: Significantly different than CaP-E-20

e: Significantly different than CaP

f: Significantly different than ACS-M1-5 $p<0.05$

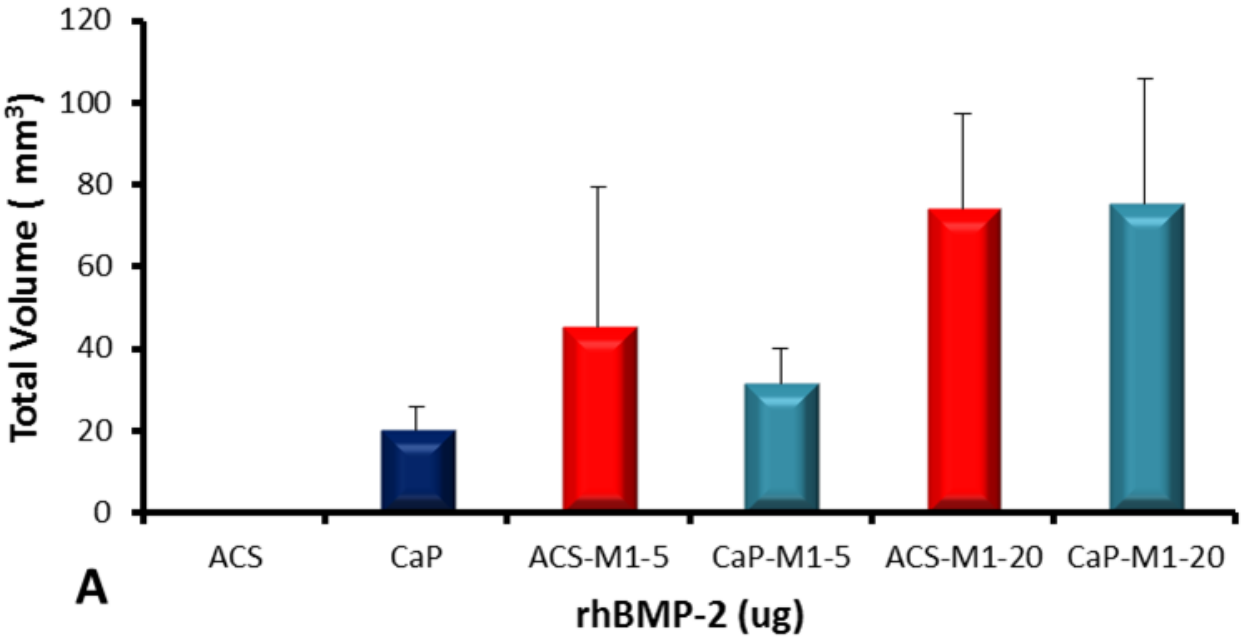
3.2.2.2 The Carrier effect on rhBMP activity

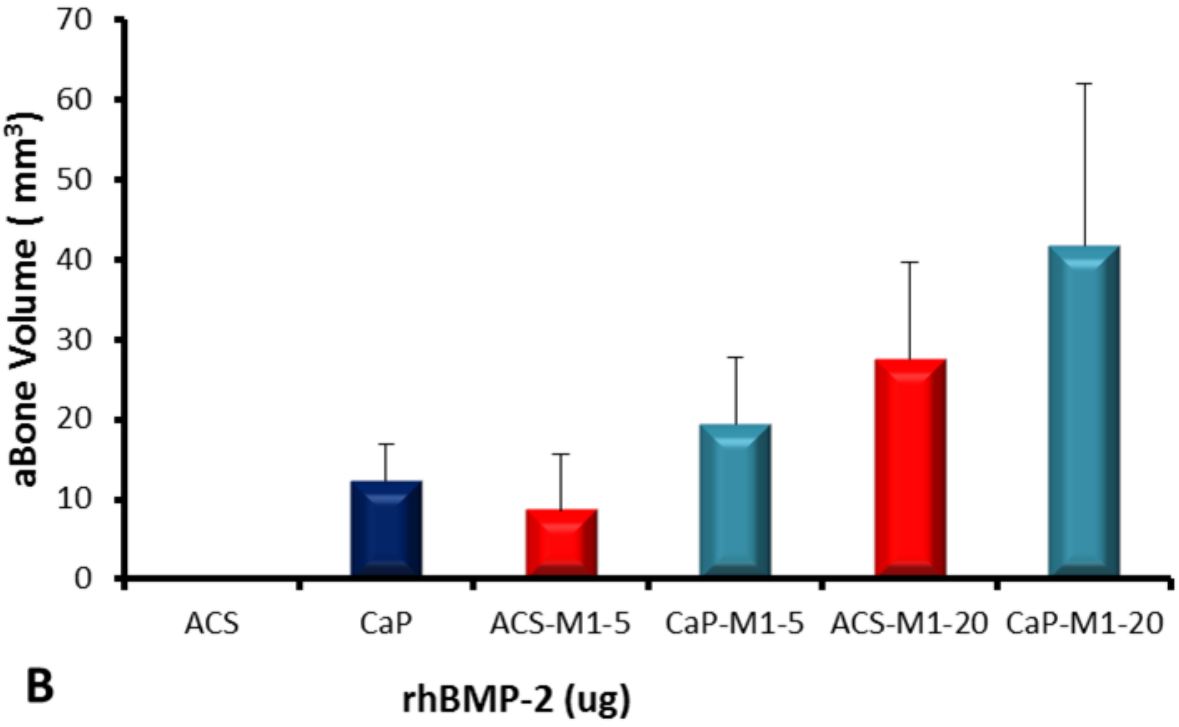
For the CHO rhBMP, the total volume, and the adjusted bone volume between the ACS and the CaP carrier were not statistically significantly different ($p>0.05$) (figures 20A, B).

For the CHO rhBMP, the adjusted tissue mineral density was statistically significantly higher on the CaP carrier than on the ACS carrier ($p<0.05$). This carrier effect was seen at both 5ug and 20ug of rhBMP ($p<0.05$). The CaP alone produced significantly higher tissue mineral density values than the CaP group on 20ug of CHO rhBMP ($p<0.05$) (figure 20C)

For the CHO rhBMP, there was no difference in bone quantity between the ACS and the CaP carrier, but the CaP carrier produced significantly higher quality of bone than the ACS carrier.

3.2.2.2.1 Comparison of ACS to CaP for the CHO rhBMP





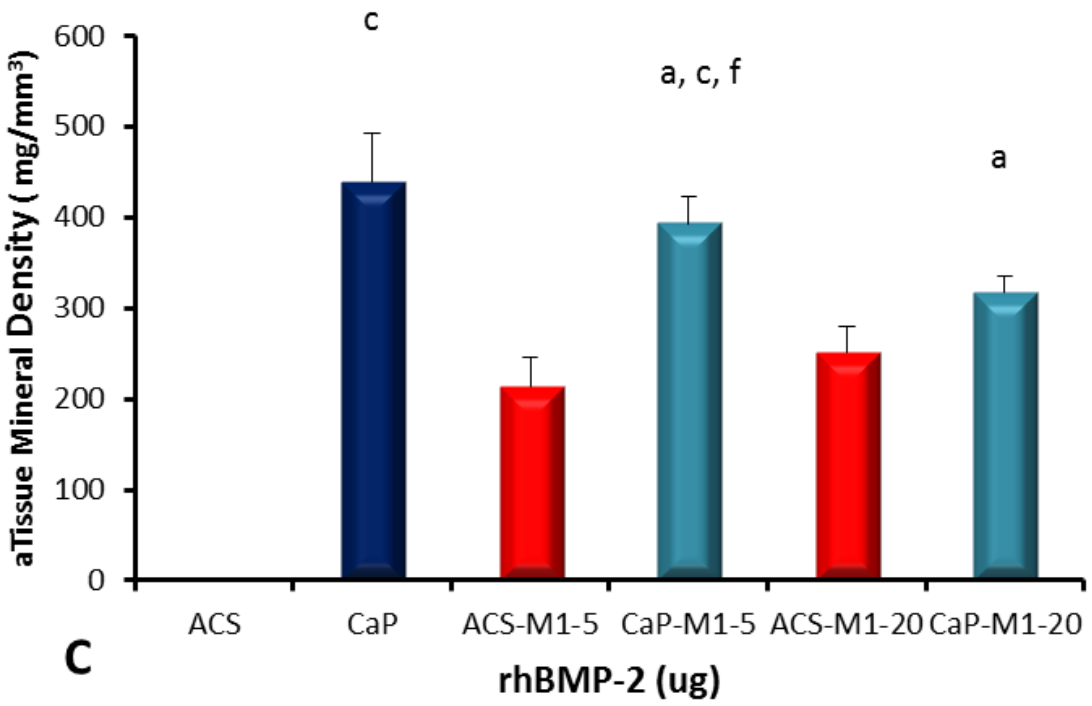


Figure 20: The difference in total volume (A), adjusted bone volume (B), and adjusted tissue mineral density (C) at 5ug and 20ug between the ACS carrier and CaP carrier for the CHO rhBMP at 28 days.

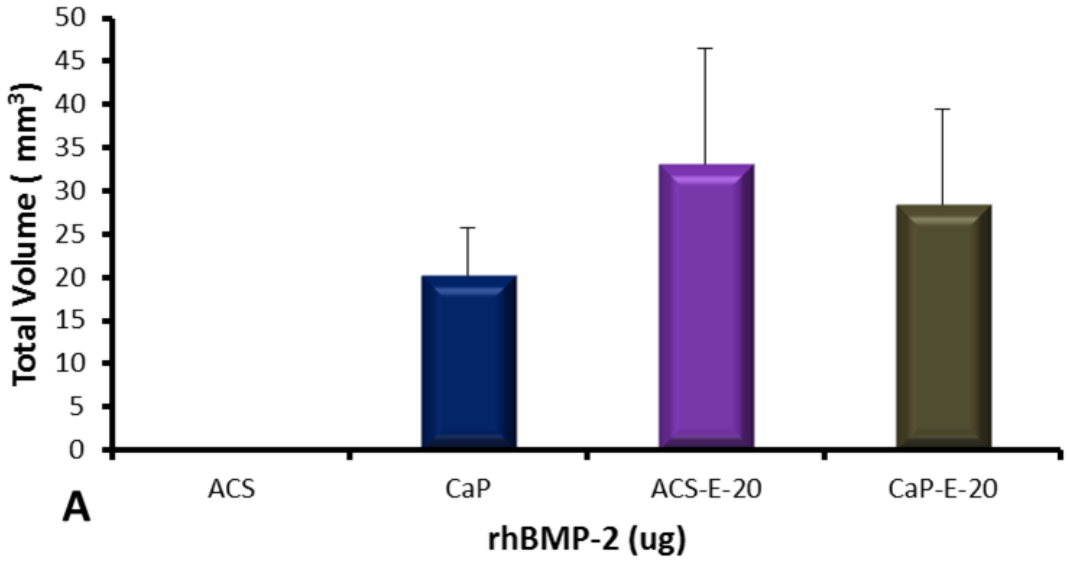
a: Significantly different than ACS-M1-20
b: Significantly different than ACS-E-20
c: Significantly different than CaP-M1-20
d: Significantly different than CaP-E-20
e: Significantly different than CaP
f: Significantly different than ACS-M1-5 $p<0.05$

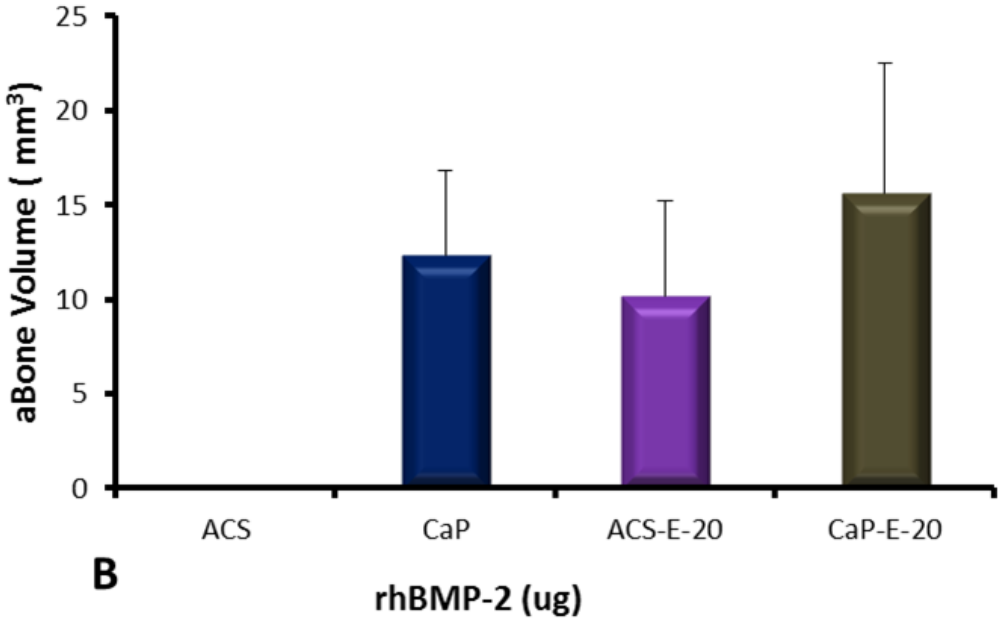
3.2.2.2.2 Comparison of ACS to CaP for the E-coli rhBMP

For the E-coli rhBMP, there was no statistically significant difference in total volume, and adjusted bone volume between the ACS group and the CaP group ($p>0.05$) (figures 21A, B).

For the E-coli rhBMP, the adjusted tissue mineral density was statistically significantly higher in the CaP group than in the ACS group ($p<0.001$) (figure 21C)

For the E-coli rhBMP, there is no statistically significant difference in bone quantity between the CaP and the ACS carrier ($p>0.05$). However, there is a statistically significant difference in the quality of bone between the CaP and the ACS carrier ($p<0.001$)


50
45
40
35
30
25
20
15
10
5
0
Total Volume (mm3)
ACS
CaP
ACS-E-20
CaP-E-20
A
rhBMP-2 (ug)


25
20
15
10
5
0
aBone Volume (mm3)
ACS
CaP
ACS-E-20
CaP-E-20
B
rhBMP-2 (ug)

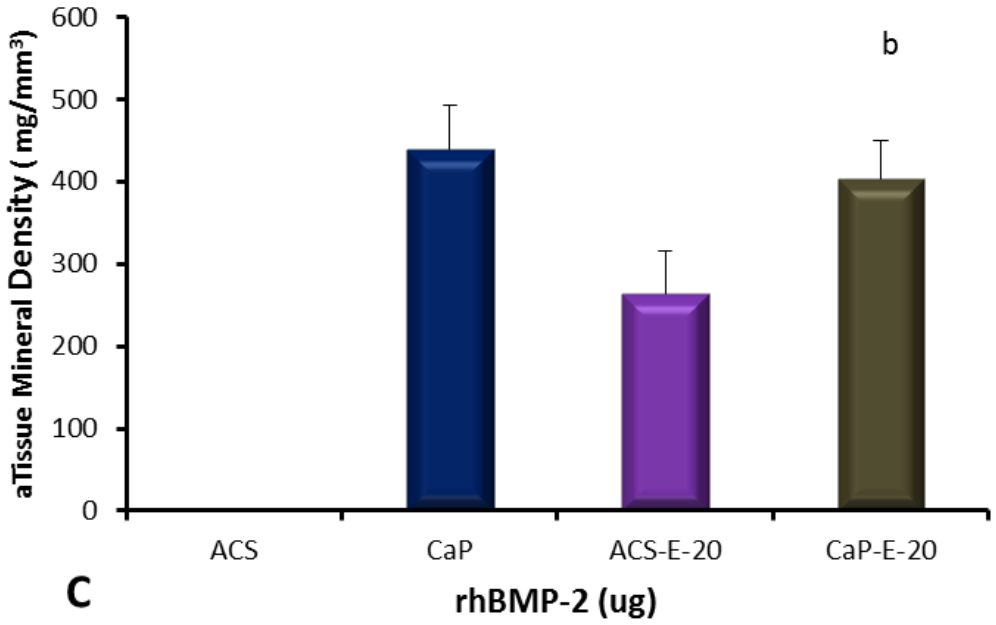


Figure 21: Difference in the total volume (A), the adjusted bone volume (B), and the adjusted tissue mineral density (C) at 20ug between the ACS carrier and CaP carrier for the E-coli rhBMP at 28 days.

a: Significantly different than ACS-M1-20

b: Significantly different than ACS-E-20

c: Significantly different than CaP-M1-20

d: Significantly different than CaP-E-20

e: Significantly different than CaP

f: Significantly different than ACS-M1-5 $p<0.05$

In summary, there was no difference in bone activity between the two CHO rhBMPs (Medtronic Infuse rhBMP and Induce rhBMP) at the doses tested (5 and 20ug).

At 20ug of rhBMP, on the CaP carrier, the CHO rhBMP produced more bone than E-coli rhBMP, but E-coli rhBMP produced higher quality bone than the CHO rhBMP.

For both the CHO and the E-coli rhBMP, the CaP carrier had a significant effect on the quality but not the quantity of bone produced versus the ACS carrier.

3.2.3 Histological Analysis

CHO and E-coli derived rhBMP-2 on the ACS induced immature woven bone with normal appearing osteocytes within lacunae, and trabeculum rimming (figures 22-26). Specimens with absorbable collagen sponge without rhBMP-2 showed no bone formation (figure 27).

When paired with the CaP granule carrier, the E-coli and CHO derived rhBMP-2 also revealed immature woven bone with normal appearing osteocytes, and peripheral trabeculum rimming (figures 28, 29). The CaP granules were visible, and appeared to have osteoblast formation at the periphery of the each granule. Unlike the absorbable collagen sponge specimen that did not contain rhBMP-2, the CaP granule specimens alone appeared to form immature woven bone with osteocytes within lacunae, and neighbouring hypertrophic chondrocytes (figure 30). Based on the histological analysis, it appeared that the bone formation in all BMP derived groups was of similar quality irrespective of the carrier and the dose used. The calcium phosphate granules alone showed osteoinductivity.

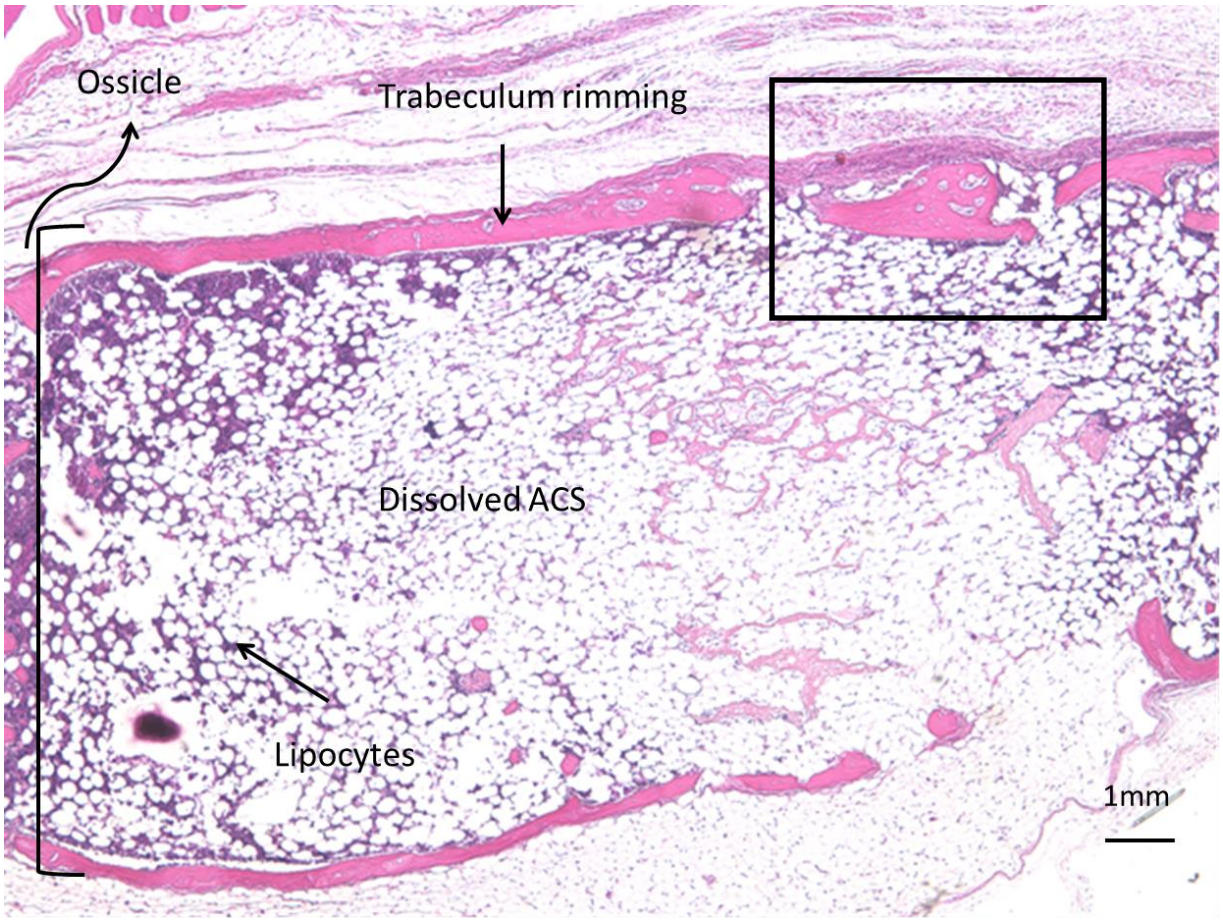


Figure 22: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from a CowellMedi BMP with 20ug of rhBMP, at low power (ACS-E-20, 4x).

The specimen shows peripheral trabeculum rimming, with immature bone trabeculae, and central lipocytes with dissolved absorbable collagen sponge.

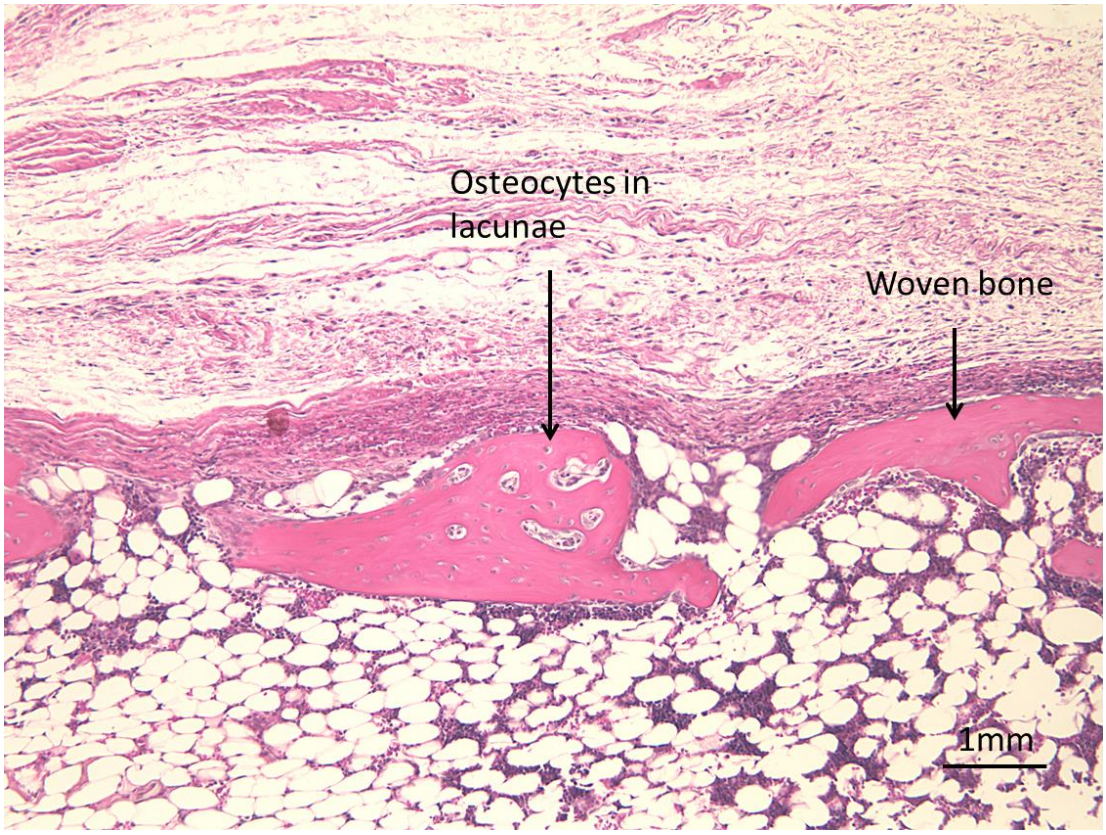


Figure 22a (inset): Photomicrograph of Hematoxylin & Eosin stained section of bone sample from a CowellMedi BMP with 20ug of rhBMP, at medium power (ACS-E-20, 10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae.

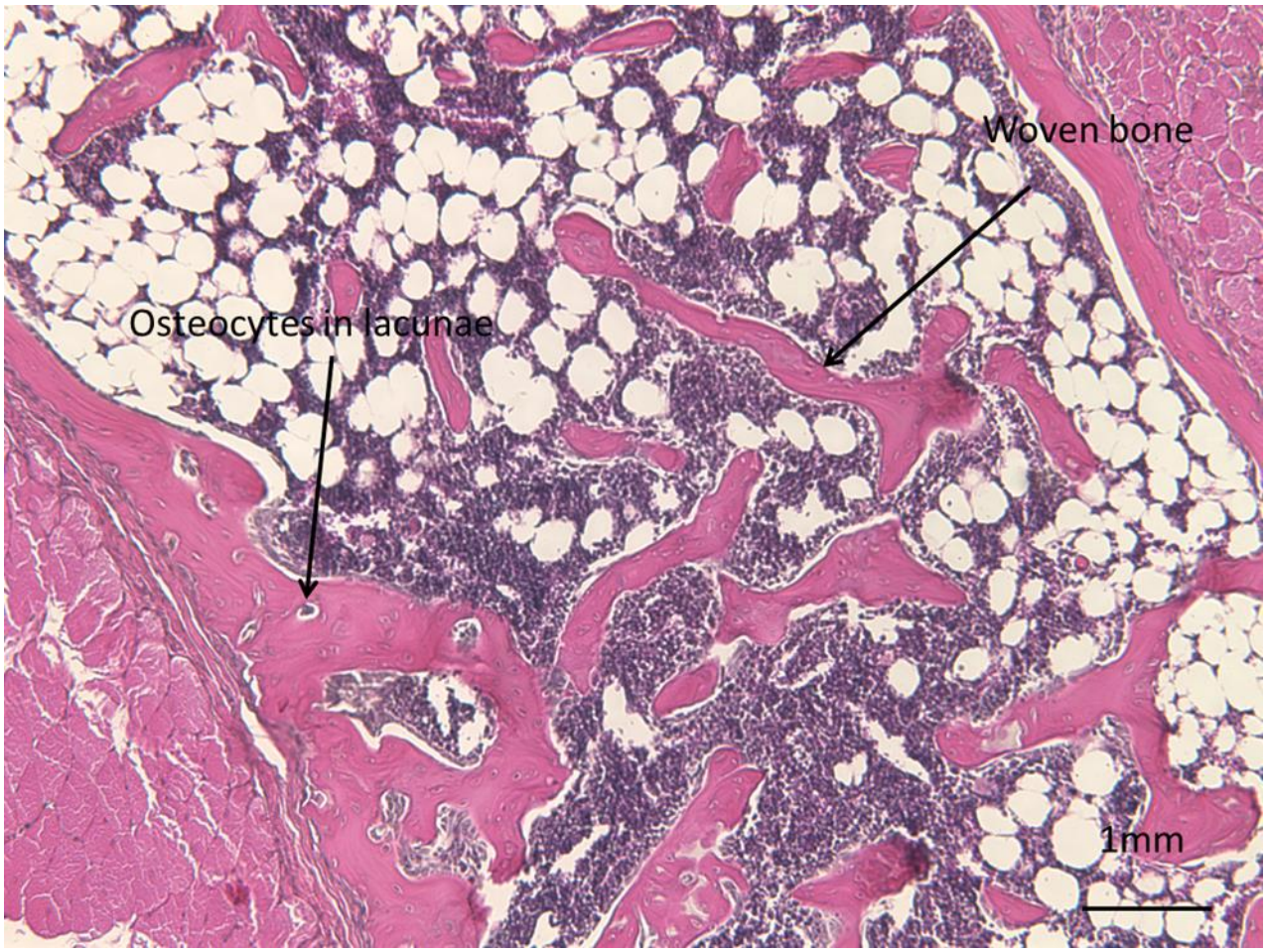


Figure 23: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from an Induce BMP with 20ug of rhBMP, at medium power (ACS-M1-20, 10x).

This section reveals a network of immature woven bone trabeculae with osteoblastic rimming, and osteocytes trapped in lacunae.

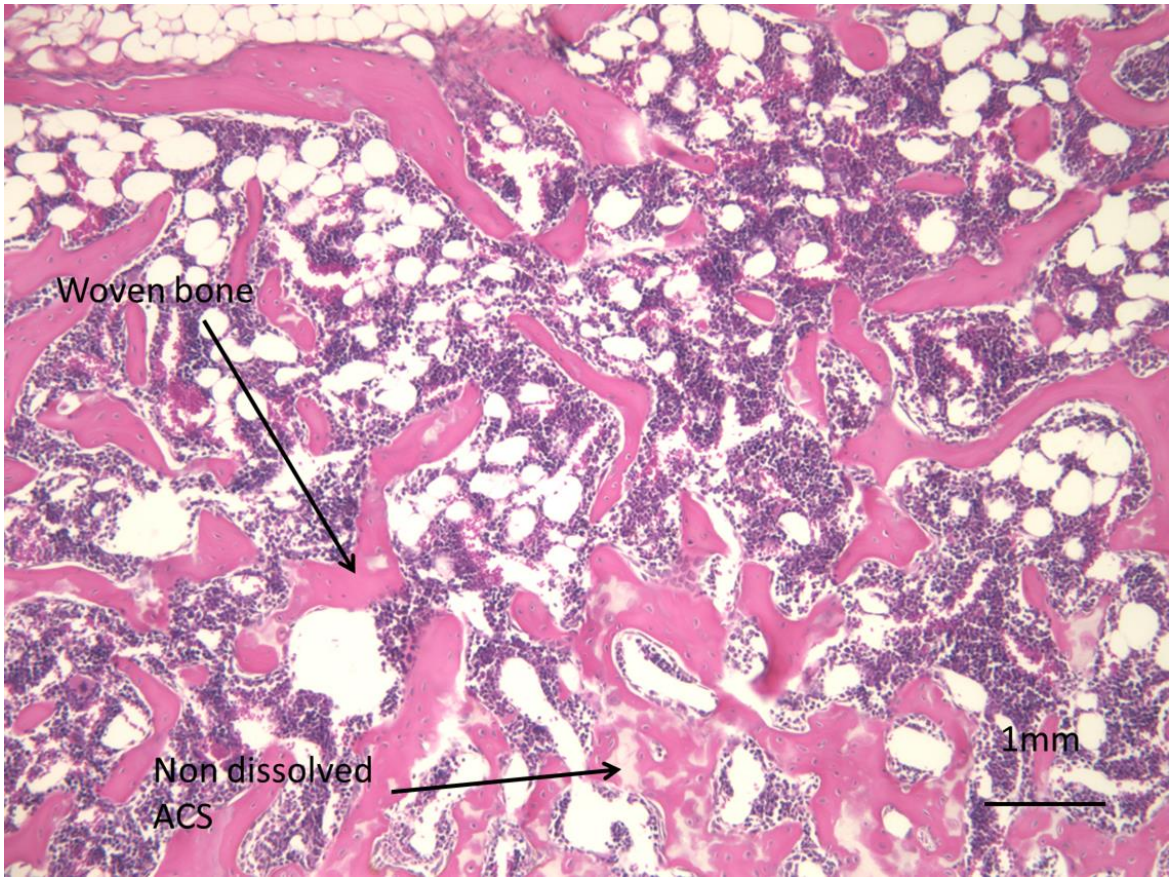


Figure 24: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from a Medtronic BMP with 20ug of rhBMP, at medium power (ACS-M2 -20, 10x).

There are areas of residual non-dissolved absorbable collagen sponge interspersed amongst the bone. This section reveals osteoblastic rimming and immature woven bone with osteocytes trapped in lacunae.

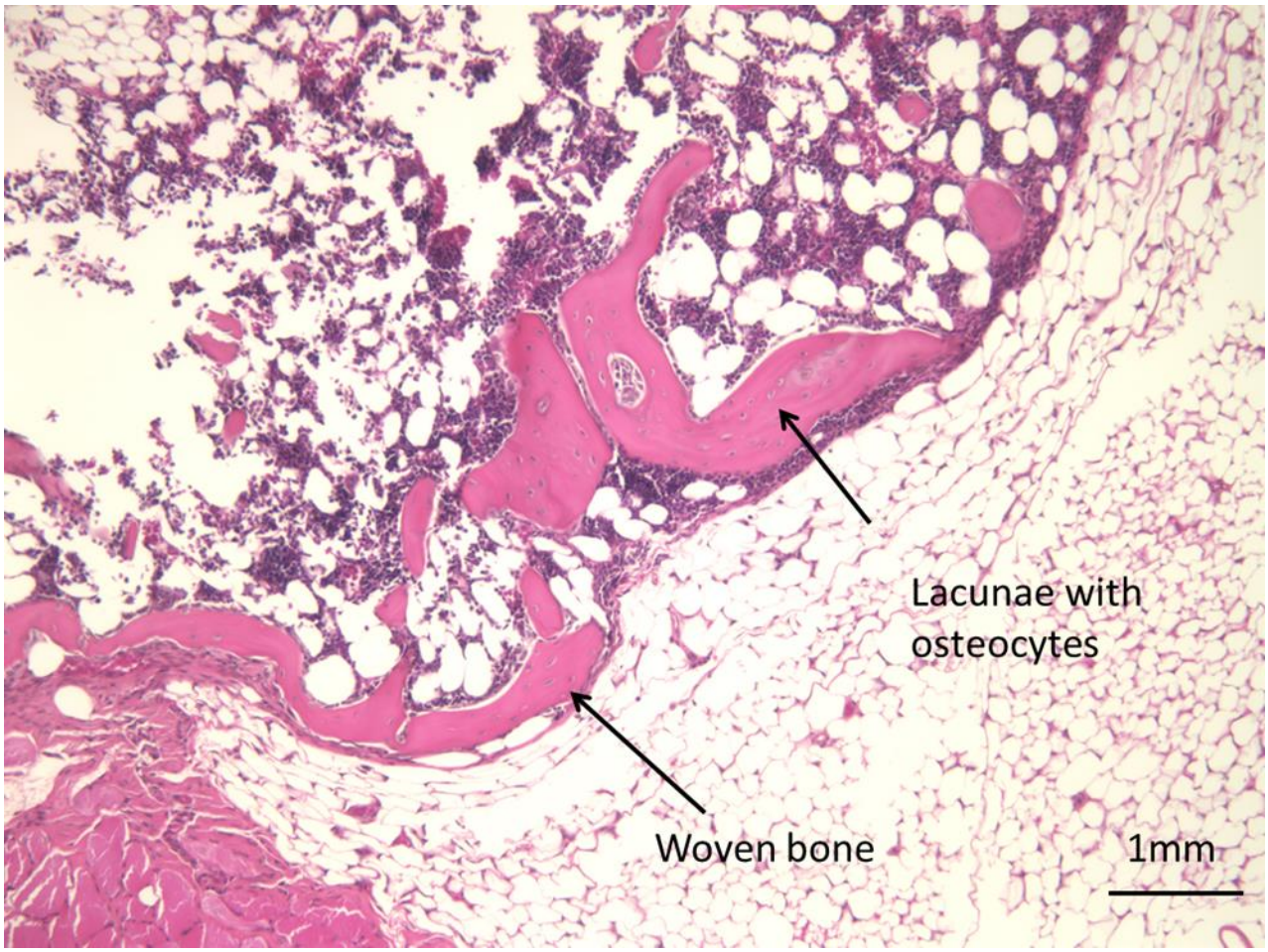


Figure 25: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from an Induce BMP at 5ug of rhBMP, at medium power (ACS-M1-5, 10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae, and osteoblastic rimming.

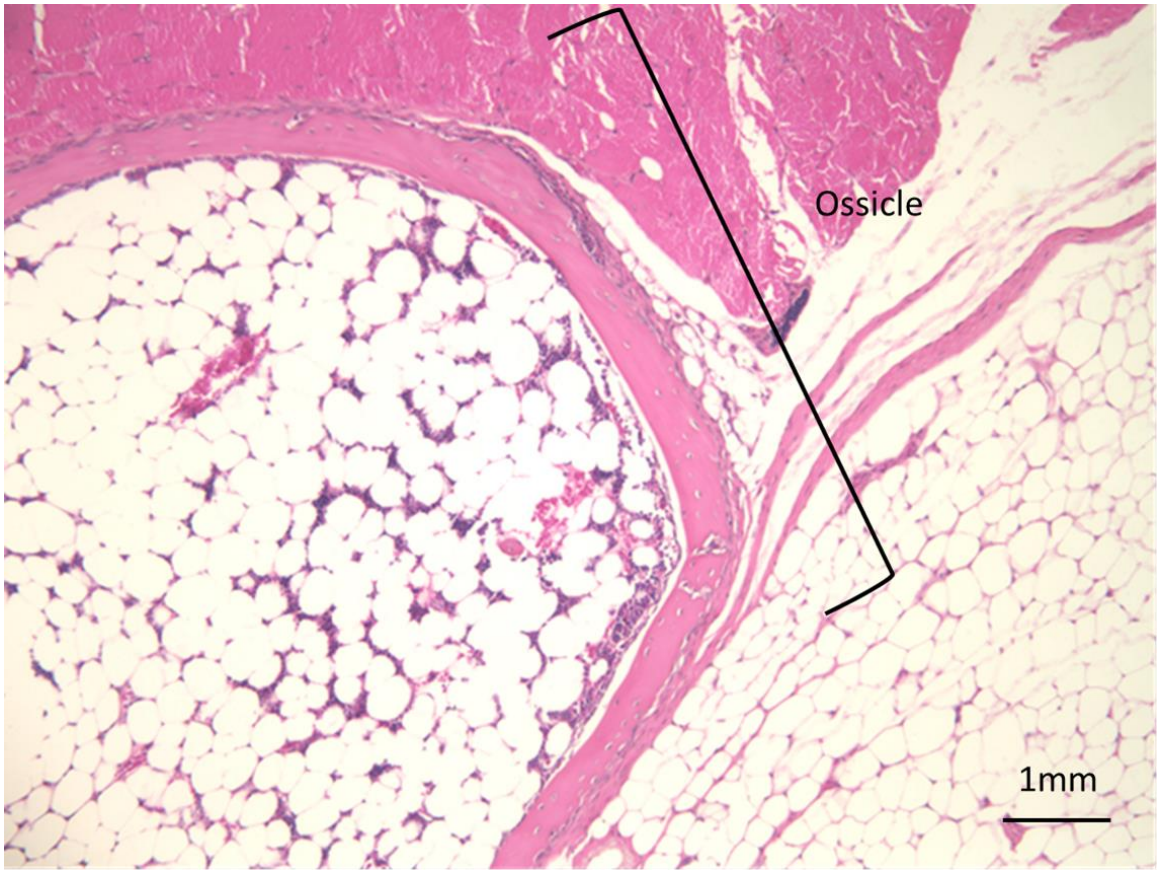


Figure 26: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from a Medtronic BMP with 5ug of rhBMP at medium power (ACS-M2-5,10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae.

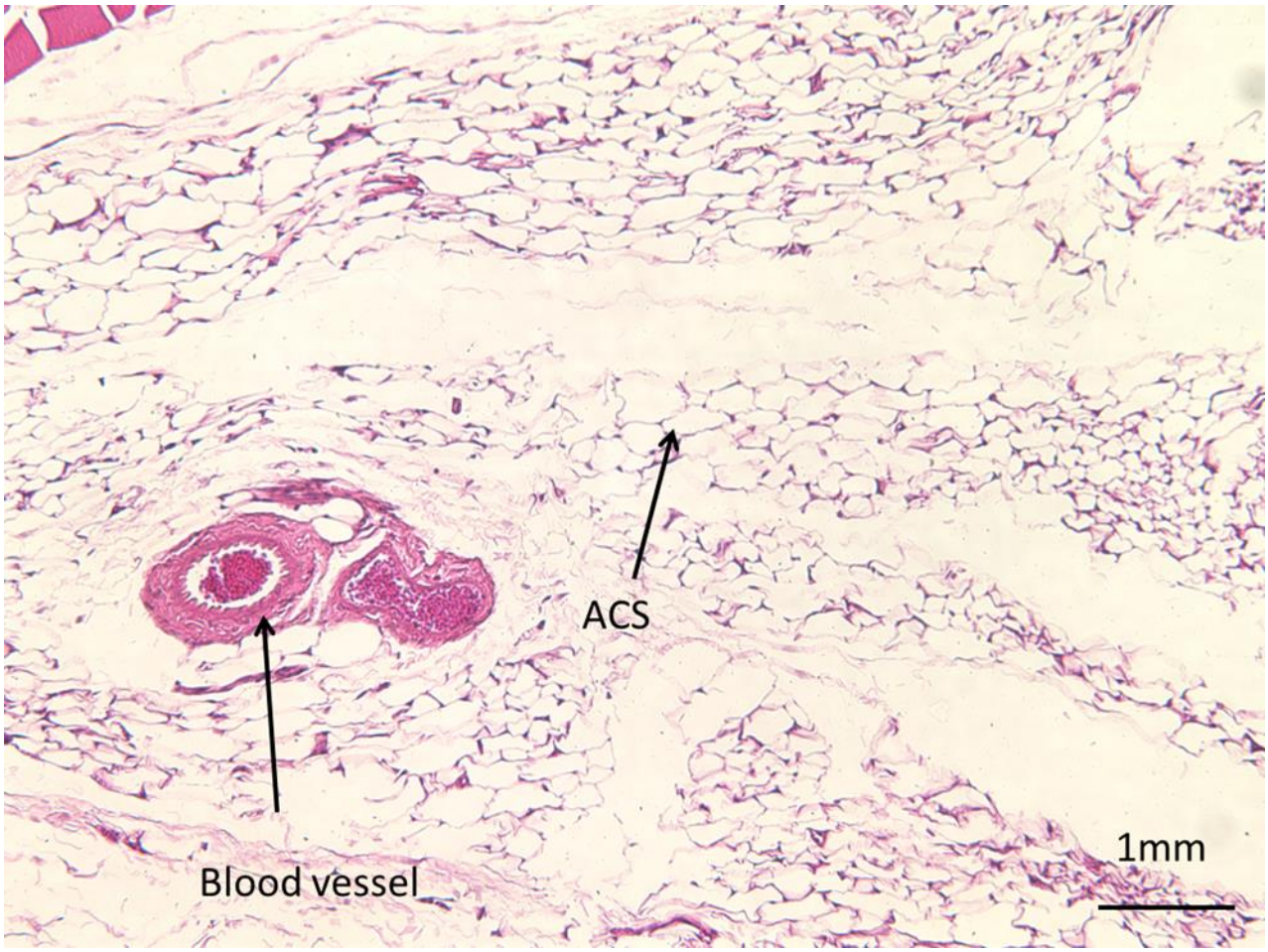


Figure 27: Photomicrograph of Hematoxylin & Eosin stained section of a sample with the absorbable collagen sponge (ACS) alone, at medium power. (ACS 10x)

There is no bone formation. There is residual ACS, and the presence of blood vessels.

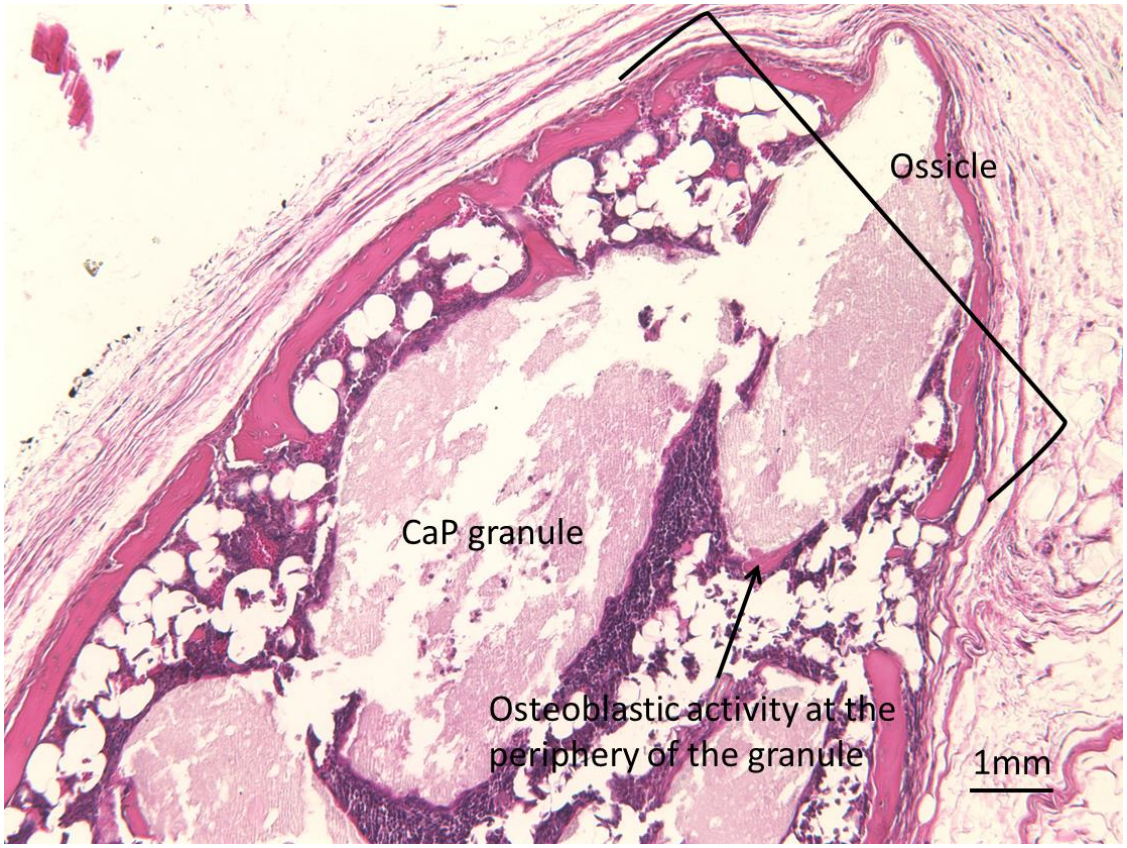


Figure 28: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from a CowellMedi rhBMP paired with the calcium phosphate granule carrier with 20ug of rhBMP at medium power (CaP-E-20, 10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae. There is evidence of osteoblastic activity at the periphery of the calcium phosphate granules.

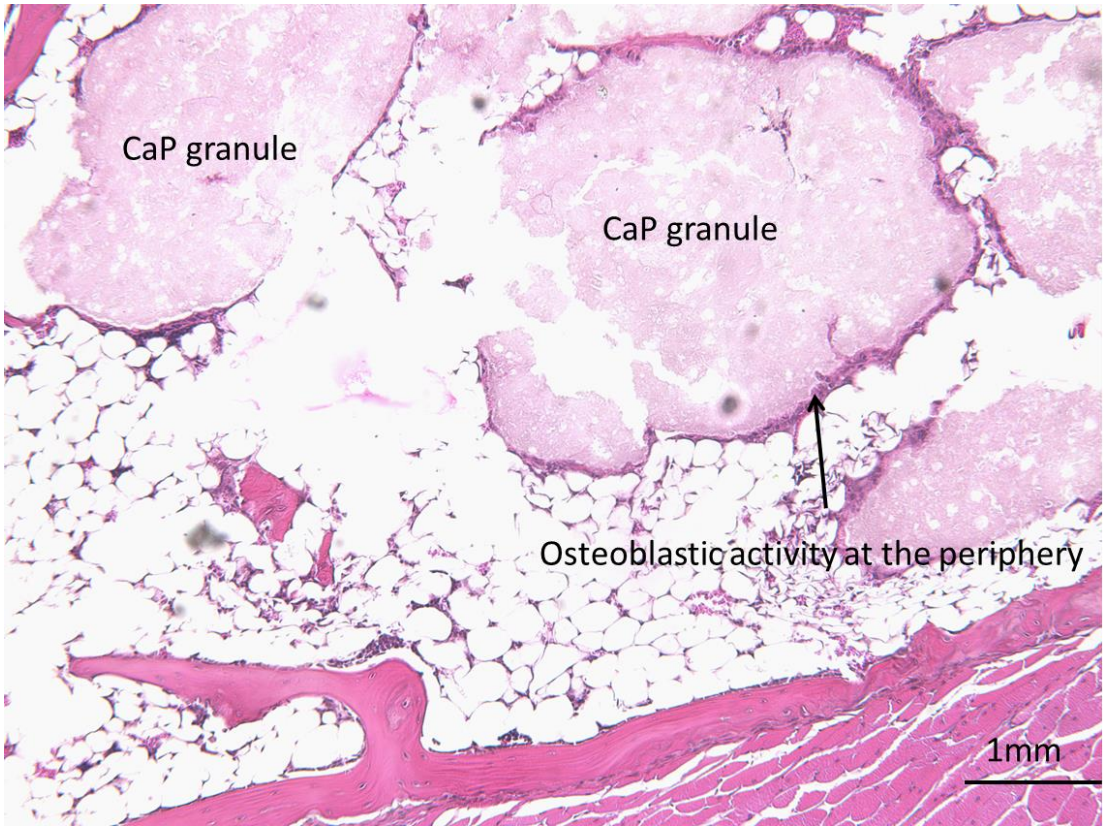


Figure 29: Photomicrograph of Hematoxylin & Eosin stained section of bone sample from an Induce rhBMP paired with the calcium phosphate granule carrier, with 20ug of rhBMP at medium power (CaP-M1-20,10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae. There is evidence of osteoblastic activity at the periphery of the calcium phosphate granules.

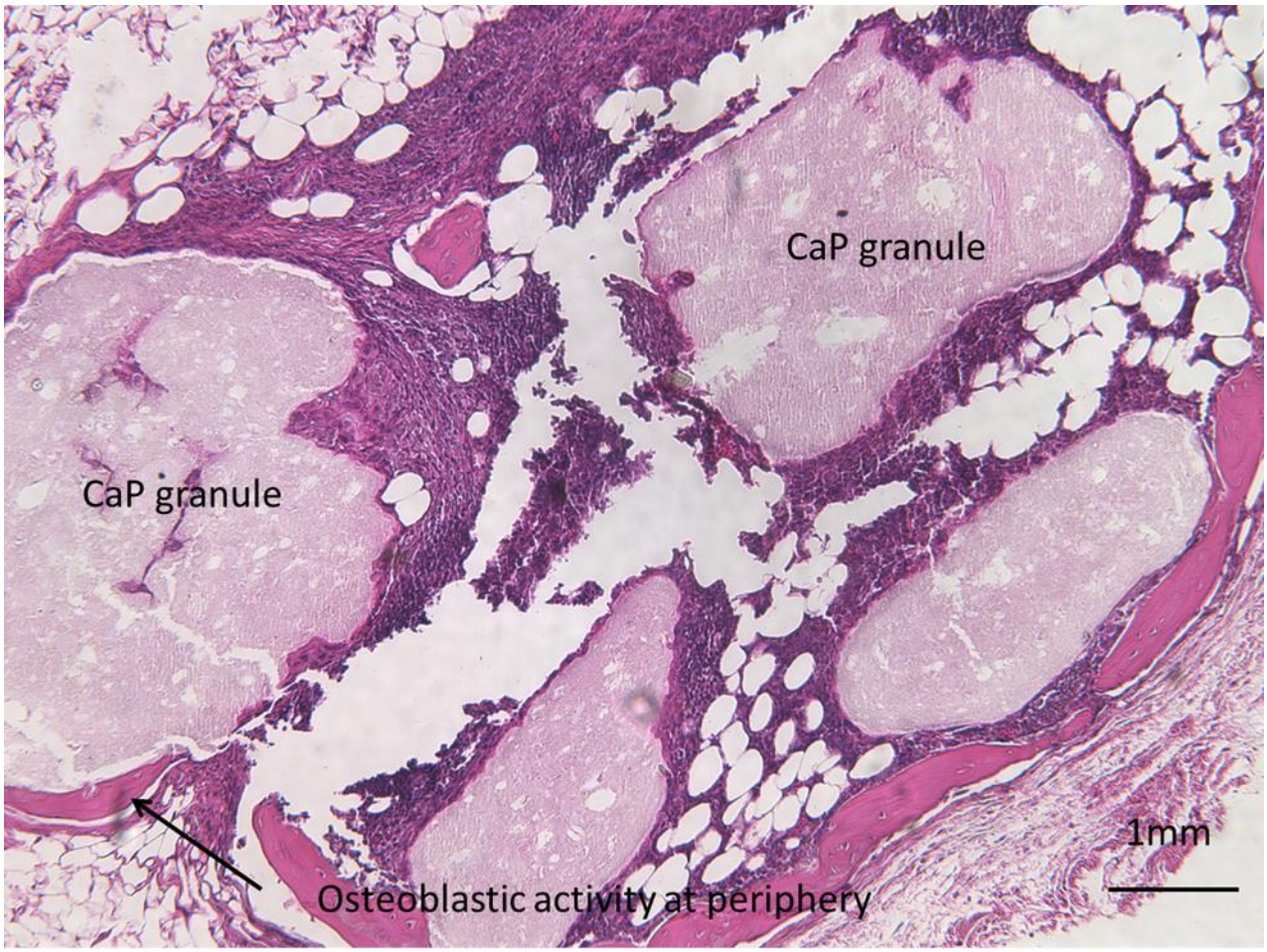


Figure 30: Photomicrograph of Hematoxylin & Eosin stained section of a sample from a calcium phosphate granule alone, at medium power (CaP-10x).

The section shows immature woven bone with normal appearing osteocytes within lacunae. There is evidence of osteoblastic activity at the periphery of the calcium phosphate granules.

4 Discussion

Efficient BMP therapy requires the development of a controlled delivery system and high-quality rhBMP. In 2002, the Medtronic Infuse CHO derived rhBMP-2 was approved by the Food and Drug Administration for clinical application in humans by delivery with a purified absorbable collagen sponge matrix [102, 219]. The use of the CowellMedi E-coli derived rhBMP-2 on the CaP granule has been approved for clinical use in Korea, and is looking to be approved in Canada. The details regarding the formulation of these bioimplants, and the specific laboratory protocols were not provided for this study beyond the information publicly available. This lack of information concerning the material formulation presents a limitation in this study. Despite this, a direct comparison of these 2 bioimplants was performed in order to evaluate the effect of the source and the carrier on rhBMP activity.

4.1 Source effect on rhBMP activity

4.1.1 In Vitro – rhBMP potency

In many studies, in vitro assays evaluated the biological activity of rhBMP-2 derived from E-coli and mammalian sources by using markers of bone cell metabolism, such as alkaline phosphatase activity, production of osteocalcin, and calcium content [46, 100, 220, 221] In the current study, the in vitro assay evaluated the activity of rhBMP-2 of both the CHO rhBMP and E-coli rhBMP by analyzing the ALP/protein activity. At all doses, and at two different time points, the results were consistent: CHO rhBMP-2 was significantly more active than E-coli rhBMP-2 in the C2C12 assay ($p<0.05$) (figure 17).

The literature supports these findings. For example, in 2002, Zhao et al found that E-coli expressed rhBMP-2 was about five to ten times less active that CHO rhBMP-2. In their study, they evaluated alkaline phosphatase activity, osteoblast proliferation, and

mineralized bone matrix formation. They attributed the difference in activity to the less efficient refolding process of rhBMP-2 protein from E-coli, which would affect its biological activity [133]. Similarly, in 2010, Einem et al studied a two-step renaturation procedure in the prokaryotic system for efficient production of recombinant BMP-2. They speculated that it was not the formation of the intramolecular cysteine knot but rather the formation of the intermolecular disulfide bond that constituted the rate limiting step, therefore causing the long renaturation times. Higher protein concentration led to dimerization or intermolecular disulfide bond formation. However, increasing the protein concentration during the renaturation process oftentimes led to protein aggregation [143]. Furthermore, in 2011, Kim et al revealed that CHO rhBMP-2 showed a significantly higher ALP activity level than E-coli rhBMP-2($p<0.001$) [146]. In 2006, Long et al found that the E-coli system produced inclusion bodies that were often inactive, not glycosylated and required extensive refolding. As these studies suggest, the highly complex refolding and renaturation processes that E-coli rhBMP-2 must undergo in order to be active may lead to the formation of proteins that are not fully functional [134, 144, 222].

Based on these studies, the most likely reason for the large difference in ALP activity between the CHO rhBMP-2 and the E-coli rhBMP-2 may be their structural differences, making the E-coli rhBMP-2 less active.

Additionally, the CHO rhBMP was prepared in a solution at a pH of 4.5, whereas the E-coli rhBMP was prepared in a solution of PBS at a pH of 7.4. It is not well known whether this difference in solution pH may have an important role to play in activity. However, it may be possible that the difference in solution pH may have contributed to the significant difference in activity in vitro between the CHO rhBMP and the E-coli rhBMP.

Many studies have used the mouse muscle C2C12 cells for in vitro evaluation of ALP activity level when exposed to rhBMP [74, 134, 136, 137, 145, 148, 149, 212, 223] . However, cell-based assays are unable to demonstrate rhBMP’s ability to induce bone, therefore, they cannot be considered the true and final tests for osteoinductivity. In order

to do so, rhBMP must be tested in vivo where the extracellular matrix, the biomechanics, the site and the dosing are more comparable to a clinical setting.

4.1.2 In Vivo

Differences in laboratory preparations have led to small differences in activity in vivo. Impurities such as viruses and bacteria, culture additives and product-related impurities can be encountered during the purification process of recombinant proteins [224] . Although not statistically significant, the differences observed in bone quantity and bone quality between the CHO rhBMPs in this study may be due to the difference in laboratory preparation (figures 18A, B, C). Based on our results, the activity of the two CHO rhBMPs was comparable on the absorbable collagen sponge carrier.

In 2000, Bessho et al performed a direct comparison of the in vitro and in vivo activity of E-coli derived rhBMP-2 to CHO derived rhBMP-2 at 2, 10 and 50ug of rhBMP mixed with 3mg of type I collagen as a carrier implanted into the calf muscle pouches of rats [148]. Similarly, in 2011, Kim et al evaluated the osteoinductivity of E-coli derived rhBMP-2 (concentrations of 5 and 50 ug) versus that of CHO derived rhBMP-2 (concentration of 10ug) on an absorbable collagen sponge carrier at 4 weeks in the mouse intramuscular tissue. Newly induced bone was evaluated using microCT reconstruction and histological analysis [146]. Kim and Bessho both found that application of E-coli rhBMP-2 on an absorbable collagen sponge showed promising activity in equivalence to CHO cell rhBMP-2 in bone tissue engineering, and that E-coli rhBMP-2 may be of value as an alternative in rhBMP therapy. They also concluded that the sufficient dose for bone induction in an ectopic site ranged from 5-20ug of rhBMP-2 on the ACS carrier [146, 148]. Therefore, the literature supports the findings of our current investigation, where the E-coli rhBMP-2 showed similar activity to CHO rhBMP-2 on the absorbable collagen sponge. However, in our study, on the CaP carrier, the CHO rhBMP showed significantly more bone activity than the E-coli derived rhBMP ($p<0.05$) (figure 19B).

Despite this, the difference in activity level between the CHO rhBMP and E-coli rhBMP, in vivo, was not as significant as the difference in their activity level in vitro. This means that the in vitro potency of the CHO and E-coli rhBMPs is not the same as their in vivo potency. Contributing factors in vivo may be the presence of the extracellular matrix, the glycosylation of the protein, and the pH of the buffer solution.

4.1.2.1 Extracellular matrix

In 2003, Peel et al used the C2C12 assay to study the activity of purified BMP and demineralized bone matrix, and the effects of the extracellular matrix on BMP activity. Their results suggested that the cellular response to BMP was enhanced through the cell-matrix receptors, such that cell interaction with collagen type I, fibronectin, and hyaluronic-coated surfaces demonstrated increased alkaline phosphatase activity. Peel's study suggested that the presence of extracellular matrix enhanced BMP cellular activity [213]. Many studies have determined that rhBMP-2 has a prolonged presence in the extracellular matrix due to the interactions with matrix components such as collagen IV, and heparin-sulfate proteoglycans, and that these interactions are critical for their osteoinductive activity [136, 225-228].

In the current study, it is possible that the activity of E-coli rhBMP increased when exposed to the extracellular matrix components, compared to its activity in vitro, which would partially explain the difference in potency in vitro versus in vivo between the 2 rhBMPs.

4.1.2.2 Glycosylation

In 1996, Ruppert et al demonstrated the interaction of rhBMP-2 with the extracellular matrix via heparin binding sites. Via these heparin binding sites, interaction of growth factors with the extracellular matrix revealed to be important in storage and stabilization of the protein, while limiting free diffusion of the factor. Their results identified the glycosylated N-terminal domain of the dimeric rhBMP-2 as a heparin-binding site that modulated biological activity, but was not obligatory for receptor activation. Given that the basic glycosylated N-terminal domains of rhBMP-2 were not

obligatory for receptor activation, post-translational glycosylation of bacterial rhBMP-2 was not as critical for a fully functional E-coli derived rhBMP-2 [136, 224].

Therefore, despite the lack of glycosylation, the suggestion is that E-coli rhBMP-2 may still lead to receptor activation, and reliable biological activity. This may be a possible cause for the difference in potency in vivo versus in vitro between the CHO rhBMP and the E-coli rhBMP observed in our study.

Glycosylation has shown to modulate protein solubility, thermostability, catalytic efficiency, antigenicity, recognition and clearance [125]. In addition to ensuring solubility, oligosaccharide structures on glycoproteins are thought to prevent aggregation of the protein [120]. For many glycoproteins, it is believed that glycosylation equates with full biological activity [134]. Glycosylated proteins, such as CHO rhBMP, are considered to be more soluble than non-glycosylated proteins, such as E-coli rhBMP[120]. This may signify that, despite the glycosylated N-terminal heparin binding site that is said to limit free diffusion of the protein, CHO rhBMP may have an increased propensity to freely diffuse away from the site of interest, therefore decreasing its biological activity. This may be another reason for the difference in potency in vivo versus in vitro between the CHO rhBMP and the E-coli rhBMP in our study.

4.1.2.3 PH of the buffer solution

In 1999, Friess et al found that a rising formulation pH led to increased incorporation of the protein onto the ACS. His findings revealed that at a pH of 4.5, significant amounts of rhBMP bound to the ACS, with further incorporation as the pH increased to 5.2, and 6.5. He concluded that pH change influences the interaction between rhBMP and collagen[183]. Given the pH of the CHO solution to be 4.5, and the pH of the E-coli formulation to be 7.4, it is possible that E-coli rhBMP had an increased interaction with the ACS versus the CHO rhBMP in vivo. This increase in ACS affinity may have led to the increase in resident time of the E-coli BMP at the site of interest, therefore lengthening its duration of effect. In contrast, with a lower formulation pH, the CHO rhBMP may have dissolved away more rapidly. This may be a reason for the

increase in E-coli rhBMP activity in vivo versus in vitro, leading to a smaller difference in activity in vivo between the E-coli and CHO rhBMP versus in vitro.

4.1.2.4 Sample size

With a sample size of n=6 per group in this study, the sample size was similar to that in other studies with similar study models [146, 148]. With a larger sample size, the results in activity in vivo between the CHO and the E-coli may have been statistically significant.

Despite the limitations of sample size, the in vitro and the in vivo results enabled the understanding of the source effect on rhBMP activity. The in vitro results demonstrated the potency of the CHO rhBMP versus that of the E-coli rhBMP in the C2C12 assay. In vitro, CHO rhBMP-2 proved to be significantly more active than E-coli rhBMP-2.The literature supports these findings. We attributed this difference in potency to the structural difference between the two rhBMPs, and the pH of the buffer solution. In accordance to the literature, our in vivo results revealed that, on the ACS carrier, the E-coli rhBMP showed similar activity to the CHO rhBMP. The difference in activity level between the CHO rhBMP and the E-coli rhBMP in vivo was not as significant as the difference in their activity level in vitro. We attributed this difference in potency in vivo versus in vitro to the presence of an extracellular matrix, the glycosylation feature of the protein, the pH of the buffer solution and the sample size. However, in vivo results also demonstrated that when paired with the CaP carrier, the CHO rhBMP produced significantly more bone than E-coli rhBMP (figure 19B), but that the E-coli rhBMP produced bone of significantly higher quality than CHO rhBMP(figure 19C). It became apparent that the carrier may have a contributing role in the difference in potency in vivo versus in vitro between the CHO and the E-coli rhBMP. Therefore, the carrier may have had an important role to play in the biologic activity of the rhBMPs in vivo.

4.2 The Carrier Effect on rhBMP activity

4.2.1 In vivo- Carrier characteristics

In 1998, De Groot et al suggested dividing carriers for BMP into 2 types: carriers that only bind a single rhBMP and carriers that concentrate the native BMP. They called them BMP carriers and BMP concentrators respectively [162]. They suggested that BMP carriers bind BMP therefor preventing free release of the protein. These would include demineralized bone matrix, collagen matrices, porous polymers and porous CaP. On the other hand, they proposed that BMP concentrators have the capacity to concentrate the native BMP to be remineralized in vivo. These would include certain calcium phosphates ceramics. In this respect, ACS may be considered a BMP carrier, while BCP may be considered a BMP concentrator. Their suggestion is that BMP retention to ACS has a more important role to play in its bioactivity, whereas certain calcium phosphate ceramics' osteoinductivity may be important in their bioactivity[162].

In the current study, the in vivo results revealed that when comparing the CHO rhBMP activity level to that of the E-coli rhBMP, CHO rhBMP produced significantly more bone than E-coli rhBMP when paired with the CaP carrier (figure 19B). On the same carrier, E-coli rhBMP produced bone of significantly higher quality than CHO rhBMP (figure 19C). Additionally, when comparing the CaP carrier to the ACS carrier on either the CHO rhBMP (figure 20), or the E-coli rhBMP (figure 21), results demonstrated that the CaP carrier had a significant effect on the quality (figures 20C, 21C), but not the quantity of bone produced versus the ACS carrier (figures 20A, B; 21 A, B). Hence, these results suggest that certain CaP carrier characteristics are more favorable to bone induction than the ACS.

To further understand the effect of the carrier on the rhBMP activity, the different carrier features were discussed, such as the method of loading of the rhBMP, the carrier geometry, the release kinetics, and the rate of degradation of the carrier.

4.2.1.1 Method of loading

In 1999, Friess et al studied the application mode of the rhBMP-2 on the collagen sponge and the amount of protein that can be mechanically expressed from the carrier. They found that with increase in soaking time, there appeared to have an increase in incorporation [154]. The recommended method of loading of the rhBMP on the ACS was to withdraw 4mL of the reconstituted rhBMP-2 from the vial and to uniformly distribute it on the collagen sponge. The wetted collagen sponge is then allowed to stand for a minimum of fifteen minutes before implantation. This method of application of the protein to the collagen sponge is what is uniformly used in other studies evaluating protein release from collagen scaffolds [199, 229]. However, in our study, the method used for loading of the rhBMP on the ACS in vivo was modified. The collagen sponge was placed in a gelatin capsule. It was then positioned in the mouse muscle pouch, and the reconstituted rhBMP-2 was pipetted into the gelatin capsule, soaking the collagen sponge. 1mg/mL of rhBMP-2 was used in vivo. At this concentration of rhBMP-2, Friess et al suggest that the soaking time should be longer to increase the potential of incorporation of the protein on the ACS carrier[154]. Also, the ACS used in the current study was sterilized with chloroform and placed under dessicator for 4 hours. It has been postulated that chloroform may damage the matrix helices. The recommended method of sterilization is gamma or electron beam radiation [156, 157].

Hence, in our study, the method of loading of the rhBMP-2 on the ACS carrier in vivo, and sterilization techniques may be contributing factors to the insignificant difference in bone quantity and quality between the CHO rhBMP and E-coli rhBMP on the ACS. The same method of loading of the rhBMP-2 on the ACS carrier was used on the CaP carrier. Unlike the ACS results, the CaP results showed significant difference in bone activity between E-coli and CHO rhBMP. Therefore, there is reason to believe that the method of loading may not be a critical factor in bone activity. Rather, the inherent characteristics of the carrier may have a more important role to play in bone activity in vivo, such as the carrier geometry, the release kinetics, and their rate of degradation.

4.2.1.2 Carrier geometry

It has been proven that larger implants allow for migration of cells and nutrients inside the implants. Smaller implants lead to more micromotion which negatively influences the process of attachment and differentiation of the cells in the center of the implant [139, 174]. With CaP ceramics, the size of the pore is believed to be more important than the actual diameter of the granule as bone formation occurs between the pores [230]. However, in a study by Le Nihouannen, the bone trabeculae were found between granules spaced less than 1 mm apart [230]. The ability for bone formation in the spaces between the CaP granules may be a contributing factor in the increase in bone activity in the CaP groups versus the ACS groups in our study.

It has been postulated that after a certain implantation time, the ectopic bone stops forming and begins to resorb, as it lacks natural mechanical strength [174, 231]. This is bound to happen earlier with the ACS carrier than with the CaP carrier, as the latter is believed to retain its mechanical strength for a longer period of time[174].

In contrast to ACS, the CaP granule has the ability to preserve space, preventing collapse of the surrounding tissue, and therefore, has an enhanced ability to maintain the rhBMP at the site of interest for a longer period of time for it to exert its biological effect. As such, the inherent space preserving ability of the CaP granules may partially explain the trend for CaP granules to form more bone then the ACS carrier in this study(figures 20B, 21B), more specifically, providing one explanation for the significant difference in bone quantity between the CHO rhBMP versus the E-coli rhBMP on the CaP carrier (figures 19A, B).

4.2.1.3 Release kinetics

Despite the high wash out effect of rhBMP in most carriers within the first few hours of placement, the short-term signal appears to be sufficient to initiate the endochondral bone cascade in various models of bone defects [214, 232, 233]. Hollinger et al reported that less than 5% of rhBMP stays at the repair site when used with a buffering system, but that use of a collagen sponge increases that retention to 15% [152]. During a 7 day period, the ACS has shown 25-40% increase in retention

compared to the initial dose[174]. Local vascularity, density of cells, local pH, ions and local clearance efficiency are all components of the microenvironment that might impact protein release [186]. For instance, between a pH of 5.0 to 7.0, there is an increase in rhBMP-2 affinity for the collagen sponge [154, 155]. However, in acidic environments, as in the hypoxic surgical wound for example, the collagen fibers cannot maintain their arrangements resulting in a softening of the collagen carrier and accelerated release of rhBMP-2[18].

In this regard, it is possible that in the surgical site, such as the wound that was created in the mouse muscle, the ACS is unable to deliver the rhBMP in a sustained fashion after the burst release. This may lead to an accelerated release of rhBMP, and a decrease in overall rhBMP activity at the site of interest. This could help explain the trend towards the increase in bone quantity in vivo between the CaP groups and the ACS on both the CHO and the E-coli rhBMP (figures 20B, 21B). In addition, this may also explain the significant difference in bone quantity in vivo between the CHO and the E-coli rhBMP on the CaP, but not on the ACS carrier (figure 19B).

Furthermore, it may be possible that, due to its slow protein release, the CaP carrier maintained the BMP at the site of interest for an extended period of time versus the absorbable collagen, thus enabling the rhBMP to exert its osteogenic potential for a longer duration. This feature is thought to be particularly useful in the ectopic site, where a higher washout rate occurs [147]. This would be one possible explanation for the significant difference in quantity of bone formed in vivo between the CHO rhBMP and the E-coli rhBMP on the CaP carrier (figure 19B). Similarly, it may also explain the trend in vivo for the CaP groups to produce more bone than the ACS groups on both the CHO and the E-coli rhBMP (figures 20B, 21B)

4.2.1.4 Rate of degradation

The collagen sponge undergoes enzymatic breakdown, versus cell-mediated breakdown that occurs with CaP granules [199]. The ACS carrier is said to have a high dissolution rate in vivo leading to lower concentrations of rhBMP at the site of interest than the CaP granules [234]. In addition to its high dissolution rate, bovine collagen-based carriers in humans have proven to develop anti-bovine collagen antibodies and

anti-human BMP antibodies, which are features that are not ideal in a carrier [113]. By virtue of its low dissolution rate, the CaP carrier should maintain its shape for a longer duration than the ACS carrier. The low dissolution rate of the CaP versus that of the ACS may signify that, at a given moment, the specific surface area of the CaP will be greater than that of the ACS. This “high” specific surface area may indicate to be essential for osteoinduction by biomaterials, as demonstrated by the increased bone activity in vivo between the CaP groups and the ACS groups.

Studies evaluating the rate of degradation of different collagen based carriers have suggested that the primary reason for rhBMP-2 retention is the three dimensional space maintenance versus the surface binding [198]. Such studies support the findings presented in our investigation, whereby, the trend was for the CaP carrier on the CHO and E-coli rhBMP to produce more bone than the ACS carrier (figures 20B, 21B).

The structural geometry, release kinetics and low dissolution rate of the calcium phosphate granules are features that may contribute to the significant difference in rhBMP activity between the CHO rhBMP and the E-coli rhBMP, in our study, when paired with the CaP granules versus the ACS. These features help understand the trend for CaP carrier to produce more bone on the CHO and E-coli rhBMP than with the ACS. In addition, these carrier features may also help clarify the reasons for the difference in potency in activity between the CHO rhBMP and the E-coli rhBMP in vivo versus in vitro.

4.2.1.5 Inherent osteoinductivity of CaP

It has been proven that, in bony sites, BCP (biphasic calcium phosphate) ceramics form strong “*direct*” bonds with the host bone resulting from a sequence of events involving the interaction with cells and the formation of carbonate hydroxyapatite by dissolution and precipitation [32, 235]. Certain bioceramics dissolve releasing calcium phosphate ions. This release of ions leads to the invasion of mesenchymal stem cells to the site, along with osteoblasts and osteoclasts to the surface of the granules. An extracellular matrix composed of collagen, non-collagenous proteins, and growth factors is formed. This matrix will later mineralize and remodel into compact bone [32, 127, 162, 174, 230, 236]. Similarly, multiple studies have shown that, in

ectopic sites, certain calcium phosphate granules have proven to form bone without the addition of an exogenous osteoinductive sources when implanted subcutaneously, by mobilizing and concentrating endogenous BMP [171, 237-239]. These studies suggest that CaP granules may act as an absorptive surface to immobilize locally produced growth and differentiation factors [162, 240]. In smaller animals, such as rats and rabbits, intramuscular and subcutaneous implantation of porous HA led to a small amount of bone formation after 45 days [32, 241]. When implanted in larger animals, such as dogs, pigs, goats and sheep, porous HA ceramics showed promising bone formation after 45 days of intramuscular implantation, and 60 days of subcutaneous implantation [241, 242]. These studies reveal that porous calcium phosphate ceramics are osteoinductive in non-bony sites, but this is dependent on animal species and type of ceramic porous structure [242].

In our study, histological analysis confirmed the presence of woven bone in the CaP samples alone (figure 30). Despite experimental methods to detach the E-coli rhBMP from the CaP granules, the lyophilized E-coli rhBMP may have remained in part attached to the granules, resulting in a false positive finding. However, it is more likely that the presence of osteoblasts and osteocytes at the surface of the CaP granules in the CaP samples signifies that the CaP carrier may be osteoinductive, as would suggest the literature. Hence, the CaP carrier may act as a protein signalling mechanism through its calcium and phosphate protein dissolution (figure 30). This inherent osteoinductive feature of the CaP granules may have an important role in the significantly higher bone quality, and the trend in increasing bone quantity, compared to the ACS, with both the E-coli rhBMP and the CHO rhBMP.

However, due to the high density of the CaP granules on microCT, it is possible that the CaP carriers were interpreted as bone during microCT analysis, despite the method described by Humber et al to distinguish bone from CaP on microCT. This hypothesis further supports our histological findings, in that, the quality of bone was similar in both rhBMP groups, regardless of the carrier used. This misinterpretation of CaP as bone may be another reason for the significant difference in bone quality between the CaP groups and the ACS groups on the CHO and the E-coli rhBMP (figures 20C, 21C). This hypothesis would explain how the CaP carrier with 5ug of CHO rhBMP showed a

statistically significantly higher quality of bone than the CaP carrier on 20ug of CHO rhBMP; the thought is that at lower doses, it would be even more difficult to distinguish bone from CaP granules (figure 21C). In addition, this may explain the significant difference observed in bone quality with the E-coli rhBMP compared to the CHO rhBMP on the CaP carrier ($p<0.05$) (figure 19C). It may also be a contributing factor in the significant difference observed in bone quantity between the CHO rhBMP and the E-coli rhBMP on the CaP carrier (figure 19A,B).

However, on both the ACS and the CaP carrier, the trend is for the CHO rhBMP to produce more bone than the E-coli rhBMP. Furthermore, it may be possible that CHO rhBMP produces more bone than E-coli, making it easier to distinguish bone from CaP on microCT; whereas E-coli rhBMP may produce less bone, making it more difficult to distinguish CaP from bone on microCT, and perhaps leading to this false interpretation of CaP as bone on microCT.

Despite the possibility of microCT misinterpretation of CaP as bone, the favorable characteristics of CaP proved to exert a more significant effect on osteoinductivity than those of the ACS (figures 20B, C, 21B, C).

Consequently, the suggestion is that CaP carrier has favorable matrix characteristics compared to the ACS scaffold in terms of structural integrity, release kinetics, and dissolution profiles, while having the potential to be inherently osteoinductive.

4.3 Relevance of the model

In addition to the source and carrier effect, bone activity has also proven to be animal dependent [230]. It is important to evaluate the adequacy of the model in addressing the aim of this study.

4.3.1 Implantation time

The standard model used in this study requires the testing at one single time-point, which is 28 days. In order to compare results with those of Kim et al, who found that the bone formation was of better quality at later than at earlier time points, it would have been ideal to have a time-point at 8 weeks [146]. The single time-point testing may be considered a limitation of this study. A second time point may have revealed more information regarding the carrier and source effect on rhBMP activity in vivo. In addition, a later time-point may have provided further information on the timing of the remodeling of the different rhBMPs.

4.3.2 Implantation site

This study used the mouse muscle pouch model which is similar to the rat subcutaneous pouch from the study by Lee et al in 2010. Limitations of this model are that, at certain anatomical sites, it does not place the bioimplant under compression, tension and shearing forces, which are important clinical parameters [147, 150].

Implantation of osteoinductive materials in extraskeletal sites has been performed in a variety of animals. Studies evaluating rhBMP-2 pharmacokinetics were done in heterotopic sites [148, 149]. Along with other authors, Lee et al found that the absorbable collagen sponge, with its limited ability to maintain space, was not favorable in orthotopic sites, where compression tends to be the primary mechanical force present [146, 147, 149]. In addition, orthotopic sites may not have exact similar responses due to different physiologic parameters [77]. In the orthotopic model, the pharmacokinetics may be affected by hematoma formation, with serum proteins prolonging in situ rhBMP-2 residence time, or serum enzymes accelerating the decay of rhBMP-2 activity [146, 147, 194, 243]. Therefore, the ectopic site is an alternative to the orthotopic site in evaluating bone activity, but does not mimic it in all aspects. This may have an effect on the ability to extrapolate results from the ectopic site model to the orthotopic clinical model.

In addition, the current study had a bilateral study design method in vivo. Although bilateral study designs allow smaller group sizes, there is potential for confounding

systemic effects of the protein being tested. Unlike the unilateral design, where the animal is more likely to favor the limb that was not surgically treated, in the bilateral design, the animal will weight bear on both limbs [231]. Therefore the mouse muscle model, with the bilateral study design method, is an adequate model used to evaluate rhBMP potential to induce bone. However, with this model, we do not expect results to be identical to those in humans. Studies performed on larger animals would be beneficial in order to more reliably evaluate the dose, and carrier effect on bone activity in vivo. Hence, the species may contribute significantly to the reliability of the results.

4.3.3 Host species

The smaller animal remains a viable option for testing bone activity in vivo, specifically if the appropriate carrier is used. In this case, the CaP carrier would be the ideal carrier to use as it has proven to have the preferred carrier characteristics for bone activity in vivo versus the ACS.

Animal model studies are at the origin of research in bone biology and in molecular biologic basis of bone healing [244]. A variety of different animals have been used to study bone repair with rhBMP including smaller animals such as the mouse, rat, and rabbit, and larger species such as the cat, the dog, the sheep, and primates. Avian animals have included chickens, and pigeons [245-249]. Individual animals with different sizes and characteristics will show the effects of different interventions on bone healing. These differences may be due to anatomic, biochemical and gene expression differences [245].

The large animal has the advantage of evaluating bone repair in relevant anatomic sites that approach the size of similar sites in humans. In addition, the large animal can help evaluate the effect of weight-bearing similarly to humans because it is not confined to a cage, like the smaller animals. However, large animals may have a smaller pool of available responsive stem cells from the surrounding bone and soft tissue envelop. Also, large animals are costly, they have the potential for disease transmission, and they are difficult to handle [95, 250].

Unlike the higher-order animals, in small animals, bone healing is achieved through a more primitive bone structure that does not include haversian systems. To date, it is not well understood whether this anatomical difference between humans and rodents is of significant importance [251]. In small animals, bone healing is believed to be much more rapid than in humans. This is thought to be related to the use of younger animals [231] The effective dose of rhBMP required in humans is very high compared to smaller animals, which suggests that the rhBMP signalling pathway is different in both species, and that bone healing in humans undergoes a slower rate of bone formation [195, 252]. Furthermore, the kinetics in humans is slightly different than that in smaller species, such that the residence time is less, and the clearance rate is higher [174]. In this regard, the preferred kinetics in rodents may not be the same as in humans, such that, slow releasing carriers are favorable in humans due to the different vascularity of the local environment and the anatomical site.

Hence, in order to counteract the discrepancies in host species characteristics, the ACS scaffold is not believed to be the preferred carrier in the human model, but rather for the mouse model. For human models, the CaP granules with its slower releasing properties, is the more ideal carrier [188, 195]. Refer to table 10

	Larger animal	Smaller animal
Compared to humans	• Simulates the effect of weight bearing • Similar anatomic sites for bone repair evaluation • Similar size	• Bone healing without haversion system • More rapid bone healing • Small rhBMP doses required • Low rhBMP clearance rate • High rhBMP residence time
Disadvantages	• Smaller pool of stem cells • Costly • Disease transmission • Difficult to handle	

Table 10: Comparison of the large animal and small animal features in experimental models.

5 Conclusion

The ability to induce bone of similar quantity and quality as native bone, from a non-autologous source, has been the challenge of reconstructive surgery in the 20th century. With the advent of growth factors and recombinant gene technology, research has led to the development of an osteogenic protein that can be produced en masse in the Chinese hamster ovary cell or E-coli bacteria. Two of the commercially available products for reconstructive surgery were compared in this study.

CHO derived rhBMP was significantly more potent than the E-coli derived rhBMP at equivalent doses in vitro. In vivo, on the CaP carrier, CHO rhBMP-2 was significantly better at inducing bone than its E-coli derived rhBMP, but the E-coli rhBMP produced bone of significantly higher quality than CHO rhBMP. Finally, the CaP carrier was more effective in the induction of bone than was the ACS carrier. Hence, the source had a significant effect on bone activity in vivo and in vitro, such that the CHO rhBMP proved to be more active than the E-coli rhBMP. In vivo, the carrier effect revealed that the CaP carrier was more conducive to bone formation than ACS. Limitations of this study include the small sample size, and the in vivo model used for the experiments. Further investigations would be necessary in order to confirm these observations, and consider these bioimplants for use in any osseous defect in humans. The findings from this study may be helpful in setting the ground determinants for these future investigations.

6 Future Development

Commercial activity is impressive in the field of improvements in bioprocess technologies for on-line monitoring of cultures. This is believed to minimize variability in metabolism across different cell culture processes [253]. Mammalian cells with robust growth and anti-apoptotic properties have been developed [254].The glycosylation apparatus of insect cells continues to catch up with mammalian cells apparatus [255]. Bacterial strains have been engineered that can facilitate the proper folding of proteins with a large number of cysteines [256].

The development of injectable carriers for the delivery of osteogenic factors for bone regeneration has been introduced for the treatment of closed fractures and for minimally invasive fracture repair. This development would lead to elimination of the need for open surgical placement of the implantable factor/carrier combination [174, 187, 194, 243].

Another novel approach to bone graft substitutes using BMP is gene therapy by integrating encoding DNA into an osteoconductive matrix such as collagen sponge [119, 257]. The goal is to allow prolonged delivery of the signal triggering bone formation. Safety and reliability of this method has yet to be demonstrated, and significant concerns exist. For a simpler and less invasive approach to delivery of osteogenic genes, a direct transfer has also been conducted. A single intralesional percutaneous injection of adenovirus with human BMP-2 cDNA was injected in a critical-size bone defect in the rat model. At 8 weeks, the site revealed healed bone [258]. Finally, a combination of rhBMPs with angiogenic factors has been attempted in order to improve the outcome by the induction of vascularized newly formed tissue [259, 260].

Research in the field of recombinant gene technology and carrier systems is continuously in the quest for the ideal product. Ongoing research is necessary to assure safety of these products in humans, and reliability of results.

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Appendix

BMP source	Dose (ng/mL)	ALP (mM) Average ± SD	p-value	Protein (ug/mL) Average ± SD	p-value	ALP/Ptn	p-value
M1	25	13.8±2	<0.0001	107.2±17.78	<0.05	0.1±0.01	<0.001
M2	25	7.2±0.2		85.3±2.8		0.1±0.001	
E	25	6.2±0.1		75.8±1.1		0.1±0.002	
M1	50	31.5±20.8	>0.05	69.2±48	>0.05	0.5±0.05	<0.05
M2	50	11.3±4.7		68.9±53.5		0.3±0.2	
E	50	5.9±0.2		76.23±11		0.1±0.01	
M1	100	37.2±39.4	<0.05	35.1±36.9	<0.05	1.087±0.3	<0.01
M2	100	67.7±6.8		137±48.7		0.5±0.2	
E	100	6±0.2		55.6±10.5		0.1±0.02	
M1	200	155.6±69.6	<0.05	69.6±21.1	<0.001	2.2±0.4	<0.001
M2	200	163.2±68.5		129.9±12.9		1.2±0.5	
E	200	5.8±0.1		45.8±5.8		0.1±0.1	
M1	400	78.01±26.6	<0.05	42.8±6.8	<0.05	1.8±0.3	<0.001
M2	400	189.2±65.9		78.3±12.3		2.4±0.6	
E	400	6±0.3		42.6±10.2		0.2±0.03	

Summary of the mean in vitro results at 30 minutes - ALP assay; M1- Induce rhBMP, M2- Infuse Medtronic rhBMP, E- CowellMedi rhBMP. ANOVA analysis with statistical significance at a level of 0.05.

		ALP	Ptn	ALP/Ptn
M1- 25 vs				
	M2- 25	<001	ns	<0.001
	E- 25	<0.001	<0.05	<0.001
M2- 25 vs				
	M1- 25	<0.001	ns	<0.001
	E-25	ns	ns	ns
E- 25 vs				
	M1- 25	<0.001	<0.05	<0.001
	M2- 25	ns	ns	ns

In vitro data post-hoc testing at 25 ng/mL of rhBMP. Significance value at 0.05

		ALP	Ptn	ALP/Ptn
M1-50 vs				
	M2- 50	ns	ns	ns
	E -50	ns	ns	<0.05
M2 -50 vs				
	M1 -50	ns	ns	ns
	E -50	ns	ns	ns
E -50 vs				
	M1- 50	ns	ns	<0.05
	M2- 50	ns	ns	ns

In vitro data post-hoc testing at 50 ng/mL of rhBMP. Significance value at 0.05

		ALP	Ptn	ALP/Ptn
M1 -100 vs				
	M2- 100	ns	<0.05	ns
	E -100	ns	ns	<0.05
M2- 100 vs				
	M1- 100	ns	<0.05	ns
	E -100	<0.05	ns	ns
E -100 vs				
	M1 -100	ns	ns	<0.05
	M2 -100	<0.05	ns	ns

In vitro data post-hoc testing at 100 ng/mL of rhBMP. Significance value at 0.05

		ALP	Ptn	ALP/Ptn
M1- 200 vs				
	M2-200	ns	<0.05	<0.05
	E- 200	<0.05	ns	<0.05
M2- 200 vs				
	M1 -200	ns	<0.05	<0.05
	E- 200	<0.05	<0.05	<0.05
E -200 vs				
	M1- 200	<0.05	ns	<0.05
	M2- 200	<0.05	<0.05	<0.05

In vitro data post-hoc testing at 200 ng/mL of rhBMP. Significance value at 0.05

		ALP	Ptn	ALP/Ptn
M1- 400 vs				
	M2- 400	<0.05	<0.05	ns
	E- 400	ns	ns	<0.05
M2- 400 vs				
	M-1 400	<0.05	<0.05	ns
	E -400	<0.05	<0.05	<0.05
E -400 vs				
	M1 -400	ns	ns	<0.05
	M2-400	<0.05	<0.05	<0.05

In vitro data post-hoc testing at 400 ng/mL of rhBMP. Significance value at 0.05

Group #	Carrier	BMP type	BMP dose (ug)	TV ±SD	aBV±SD	BMC±SD	BMD±SD	aTMC±SD	aTMD±SD	aBVF±SD
1	ACS	---	0	0	0	0	0	0	0	0
2	ACS	M1	5	45.2±34.1	8.6±7	1.6±1.8	41.2±28.5	1.9±1.8	213.3±33.6	0.2±0.1
3	ACS	M1	20	74.04±23.4	27.5±12.2	7.1±4.5	98.4±46.5	7.1±3.7	251.03±29.6	0.4±0.1
4	ACS	M2	5	23.4±14.1	7.06±4.3	1.6±1	73.7±34.6	1.6±1	225.5±33.5	0.3±0.1
5	ACS	M2	20	44.6±22.6	19.4±11.3	5.2±3.1	120±46.4	4.9±3	251.8±9.3	0.5±0.2
6	ACS	E	20	28.8±15.9	8.8±5.7	2.2±1.9	84.5±67	2.3±1.6	263.7±47.1	0.3±0.2
7	CaP	---	0	20.1±5.6	12.3±4.6	11.4±1.8	586.6±114	5.3±1.5	439.5±53.3	0.6±0.1
8	CaP	M1	5	31.5±8.7	19.3±8.4	13.5±3.2	437.6±67.4	7.6±3.5	393.4±30.2	0.6±0.2
9	CaP	M1	20	75.4±30.6	41.8±20.2	20.04±6.4	272.7±42.3	13.1±6	317.2±18.7	0.6±0.1
10	CaP	E	20	28.4±11.1	15.7±6.9	12.6±2.9	473.1±107.2	6.1±2.2	402.8±47.6	0.6±0.1
ANOVA Pvalue	------	-----	-----	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

In vivo data of all experimental groups at 28 days. Standard deviations of all 7 bone parameter values are noted. ANOVA with p values included.M1- Induce rhBMP, M2- Medtronic rhBMP, E- CowellMedi rhBMP, CaP- calcium phosphate, ACS- absorbable collagen sponge.

ACS vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
CaP	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
ACS-M1-5	<0.007	ns	ns	ns	ns	<0.0001	ns
ACS-M2-5	ns	ns	ns	ns	ns	<0.0001	<0.001
CaP-M1-5	ns	<0.05	<0.0001	<0.0001	<0.003	<0.0001	<0.0001
CaP-M1-20	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CaP-E-20	ns	ns	<0.0001	<0.0001	<0.05	<0.0001	<0.0001
ACS-E-20	ns	ns	ns	ns	ns	<0.0001	<0.0001
ACS-M1-20	<0.0001	<0.001	<0.05	ns	<0.007	<0.0001	<0.0001
ACS-M2-20	<0.008	<0.05	ns	ns	ns	<0.0001	<0.0001
CaP vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
ACS	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
ACS-M1-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
ACS-M2-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.002
CaP-M1-5	ns	ns	ns	<0.009	ns	ns	ns
CaP-M1-20	<0.0001	<0.0001	<0.001	<0.0001	<0.002	<0.0001	ns
CaP-E-20	ns	ns	ns	ns	ns	ns	ns
ACS-E-20	ns	ns	<0.001	<0.0001	ns	<0.0001	<0.003
ACS-M1-20	<0.001	ns	ns	<00001	ns	<0.0001	<0.05
ACS-M2-20	ns	ns	ns	<0.0001	ns	<0.0001	ns
ACS-M1-5 vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
ACS	<0.007	ns	ns	ns	ns	<0.0001	ns
CaP	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001

	ACS-M2-5	ns	ns	ns	ns	ns	ns	ns
	CaP-M1-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
	CaP-M1-20	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	CaP-E-20	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
	ACS-E-20	ns	ns	ns	ns	ns	ns	ns
	ACS-M1-20	ns	ns	ns	ns	ns	ns	ns
	ACS-M2-20	ns	ns	ns	ns	ns	ns	<0.05
ACS-M2-5 vs		TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
	ACS	ns	ns	ns	ns	ns	<0.0001	<0.001
	CaP	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.002
	ACS-M1-5	ns	ns	ns	ns	ns	ns	ns
	CaP-M1-5	ns	ns	<0.0001	<0.0001	<0.05	<0.0001	<0.002
	CaP-M1-20	<0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.001	<0.05
	CaP-E-20	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.05
	ACS-E-20	ns	ns	ns	ns	ns	ns	ns
	ACS-M1-20	<0.001	<0.05	ns	ns	ns	ns	ns
	ACS-M2-20	ns	ns	ns	ns	ns	ns	ns
CaP-M1-5 vs		TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
	ACS	ns	<0.05	<0.0001	<0.0001	<0.003	<0.0001	<0.0001
	CaP	ns	ns	ns	<0.009	ns	ns	ns
	ACS-M1-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
	ACS-M2-5	ns	ns	<0.0001	<0.0001	<0.05	<0.0001	<0.002
	CaP-M1-20	<0.05	<0.05	<0.05	<0.002	ns	<0.05	ns

	CaP-E-20	ns	ns	ns	ns	ns	ns	ns
	ACS-E-20	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.003
	ACS-M1-20	<0.05	ns	ns	<0.0001	ns	<0.0001	ns
	ACS-M2-20	ns	ns	<0.003	<0.0001	ns	<0.0001	ns
CaP-M1-20 vs		TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
	ACS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	CaP	<0.0001	<0.0001	<0.001	<0.0001	<0.002	<0.0001	ns
	ACS-M1-5	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	ACS-M2-5	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.001	<0.05
	CaP-M1-5	<0.05	<0.05	<0.05	<0.002	ns	<0.05	ns
	CaP-E-20	<0.004	<0.001	<0.05	<0.0001	<0.007	<0.003	ns
	ACS-E-20	<0.005	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.05
	ACS-M1-20	ns	ns	<0.0001	<0.001	<0.05	ns	ns
	ACS-M2-20	ns	<0.05	<0.0001	<0.007	<0.001	ns	ns
CaP-E-20 vs		TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
	ACS	ns	ns	<0.0001	<0.0001	<0.05	<0.0001	<0.0001
	CaP	ns	ns	ns	ns	ns	ns	ns
	ACS-M1-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
	ACS-M2-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.05
	CaP-M1-5	ns	ns	ns	ns	ns	ns	ns
	CaP-M1-20	<0.004	<0.001	<0.05	<0.0001	<0.007	<0.003	ns
	ACS-E-20	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.05
	ACS-M1-20	<0.006	ns	ns	<0.0001	ns	<0.0001	ns
	ACS-M2-20	ns	ns	<0.05	<0.0001	ns	<0.0001	ns

ACS-E-20 vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
ACS	ns	ns	ns	ns	ns	<0.0001	<0.0001
CaP	ns	ns	<0.001	<0.0001	ns	<0.0001	<0.003
ACS-M1-5	ns	ns	ns	ns	ns	ns	ns
ACS-M2-5	ns	ns	ns	ns	ns	ns	ns
CaP-M1-5	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.003
CaP-M1-20	<0.005	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.05
CaP-E-20	ns	ns	<0.0001	<0.0001	ns	<0.0001	<0.05
ACS-M1-20	<0.007	ns	ns	ns	ns	ns	ns
ACS-M2-20	ns	ns	ns	ns	ns	ns	ns
ACS-M1-20 vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
ACS	<0.0001	<0.001	<0.05	ns	<0.007	<0.0001	<0.0001
CaP	<0.001	ns	ns	<0.0001	ns	<0.0001	<0.05
ACS-M1-5	ns	ns	ns	ns	ns	ns	ns
ACS-M2-5	<0.001	<0.05	ns	ns	ns	ns	ns
CaP-M1-5	<0.05	ns	ns	<0.0001	ns	<0.0001	ns
CaP-M1-20	ns	ns	<0.0001	<0.001	<0.05	ns	ns
CaP-E-20	<0.006	ns	ns	<0.0001	ns	<0.0001	ns
ACS-E-20	<0.007	ns	ns	ns	ns	ns	ns
ACS-M2-20	ns	ns	ns	ns	ns	ns	ns
ACS-M2-20 vs	TV	aBV	BMC	BMD	aTMC	aTMD	aBVF
ACS	<0.008	<0.05	ns	ns	ns	<0.0001	<0.0001
CaP	ns	ns	ns	<0.0001	ns	<0.0001	ns

	ACS-M1-5	ns	ns	ns	ns	ns	ns	<0.05
	ACS-M2-5	ns	ns	ns	ns	ns	ns	ns
	CaP-M1-5	ns	ns	<0.003	<0.0001	ns	<0.0001	ns
	CaP-M1-20	ns	<0.05	<0.0001	<0.007	<0.001	ns	ns
	CaP-E-20	ns	ns	<0.05	<0.0001	ns	<0.0001	ns
	ACS-E-20	ns	ns	ns	ns	ns	ns	ns
	ACS-M1-20	ns	ns	ns	ns	ns	ns	ns

In vivo post Hoc testing of all data. 7 bone parameters. Significance at the 0.05 level.